

STRUCTURAL STUDIES ON THE
ALGAL BILIPROTEINS

by

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I should like to take this opportunity to express my thanks to Professor Sir Edmund Hirst, O.B.E., F.R.S., and the University of Edinburgh for providing the laboratory facilities for my research; to Dr. R.A. Wall for all his guidance and help; and to my colleagues who have made the last three years a most enjoyable experience. In particular I should like to thank Mr. R.P. Salmon, B.Sc., for his work on the isolation of C-phycoerythrin.

TO MY PARENTS
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Three methods of degradation were used - acidic, basic and enzymic hydrolyses. Acidic hydrolysis was carried out by the method of O'Garra (Doctoral Thesis, National University of Ireland) in which a large excess of ethyl mercaptan competes with protein fragments for the pigment released by the acid, forming a chloroform-soluble material. Silica-gel chromatography showed that this material was a mixture of a number of coloured components, but there was insufficient material available to permit complete nuclear magnetic resonance analysis of the major component.

SUMMARY

The blue-green alga *Anabaena cylindrica* was grown in a medium containing sodium, magnesium, calcium, phosphate and trace amounts of ten other elements. Cultures were illuminated by a fluorescent source and maintained at 24°C; air containing 5% carbon dioxide was bubbled through the medium, and the presence of glass wool was found to increase the growth rate by providing a greater surface area. The algal cells were ruptured by sonication and the crude protein thus released was precipitated with ammonium sulphate; it was then redissolved in water, and the solution was filtered through a pad of celite and purified by basic calcium phosphate chromatography. The purity of this C-phycocyanin was demonstrated by its spectral properties, by disc electrophoresis and by chromatography on Sephadex. Crystallisation of the protein from a buffer solution containing ammonium sulphate yielded needle-shaped crystals. Carbohydrate analysis, carried out by the Phenol-Sulphuric Acid method, showed that the protein contained approximately 1% carbohydrate.

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Basic hydrolysis of the protein was found to bring about extensive cleavage of the molecule as shown by chromatography and electrophoresis but there was no evidence to suggest cleavage of the chromophore.

Enzymic hydrolyses were found to give the most interesting results. Trypsin brought about a degree of cleavage of the molecule which was of the order predicted on the basis of the number of lysine and arginine residues present, Trypsin being specific for peptide bonds activated by these residues. Pronase brought about a greater degree of cleavage; Sephadex chromatography of digests led to the separation of a large number of components, the largest having a molecular weight of around 13,000. The fraction of greatest interest was that eluted from the Sephadex columns by 50% aqueous acetic acid; this showed fluorescence under ultraviolet light, and this and other evidence suggested that it was a chromopeptide. Treatment of this material with phenyl isothiocyanate followed by reduction with lithium borohydride and analysis showed the presence of large amounts of proline, which would be expected if a ω -glutamic ester were present. Similar results were obtained on reduction of peptides extracted by amyl alcohol from Pepsin digests of C-phycocyanin; also the changes in composition of these peptides after successive stages of purification suggested that the chromophore is located in a hydrocarbon-like region of the protein molecule.

Methanolysis of the protein showed that a pigment thus released which had been thought to be a pure chromophore in fact contained a small amount of peptide material. The

compositions of this material and the chromopeptides obtained by enzymic digestion were similar in several respects; these results were interpreted as showing that aspartic acid, threonine, serine and glutamic acid were present in equimolar quantities in the chromopeptide, the linkage to the chromophore being through the ω -carboxyl group of glutamic acid; also that the hydrocarbon sheath around the chromophore contained valine, isoleucine, leucine and phenyl alanine in the ratio 3:3:2:1.5.

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INTRODUCTION

The algal chromoproteins are coloured proteins found in certain groups of algae - namely the divisions Rhodophyta, Cyanophyta and Cryptophyta, otherwise known as, respectively, Red, Blue-green and Cryptomonad algae. These algae, which all belong to the Thallophyta, provide the only known sources of these proteins, save for some members of the Division Chlorophyta. One of these proteins, phycocyanin, has also been reported in the coastal lichen, *Lichina Pygmaea* (Quillet, 1961). The algal chromoproteins are related to the protein phytochrome, a chromoprotein of higher plants, particularly by the similarity between their prosthetic groups, which was confirmed by Siegelman (1965).

Those algae whose biliproteins have been studied are listed in Table I. The proteins are located in the lamellae of chloroplasts of Red Algae (Haurowitz, 1958). Electron Micrograph studies indicate that Blue-green algae also contain chloroplast-like structures (Elbers et al., 1957), and it seems likely that the chromoproteins are located in their lamellae, rather than in free chromatophores or in the cytoplasm (Thomas and de Rover, 1955).

A phycocyanin was the first algal biliprotein to be studied. In 1836, Von Esenbeck of Bonn reported that a blue colouring matter, probably related to the proteins, was released on autolysis of an *Oscillatoria* species. Many later workers referred to the beauty of the transmitted and fluorescent colours of the algal chromoproteins, e.g. Sorby (1877). These proteins have found little practical use

TABLE I. Distribution of the Algal Biliproteins.

<u>RHODOPHYTA</u>		
<u>CLASS: Bangiophyceae</u>		
<u>ORDER: Porphyridiales.</u> <i>Porphyridium cruentum</i>	B-PE; R-PC; Allo-PC.	Haxo et al., 1955; O'hEocha, 1955
<u>ORDER: Bangiales</u> <i>Porphyra tenera</i> <i>Porphyra perforata</i> <i>Smithora naiadum</i>	R-PE; C-PC; Allo-PC R-PE; R-PC; Allo-PC. B-PE; C-PC; Allo-PC	Hattori and Fujita (1959). Jones and Blinks (1957). Airth and Blinks (1956)
<u>CLASS: Florideophyceae</u>		
<u>ORDER: Nemalionales</u> <i>Rhodochorton rothii</i> <i>Rhodochorton floridulum.</i>	R-PE; PC B-PE; PC	O'hEocha (1958). O'hEocha and O'Carra (1961)
<u>ORDER: Gigartinales</u> <i>Plocamium pacificum</i>	R-PE	O'hEocha (1958).
<u>ORDER: Cryptonemiales</u> <i>Grateloupia sp.</i>	R-PE; C-PC; AlloPC.	Hattori and Fujita (1959).
<u>ORDER: Rhodymeniales</u> <i>Rhodymenia palmata</i>	R-PE; R-PC; AlloPC.	O'hEocha, 1960.
<u>ORDER: Ceramiales</u> <i>Ceramium rubrum</i> <i>Polysiphonia urceolata</i>	R-PE; R-PC; AlloPC R-PE; C-PC; Allo PC	Svedberg and Katsurai (1929) Hattori and Fujita (1959).
<u>CYANOPHYTA</u>		
<i>Tolypothrix tenuis</i> <i>Arthrospira maxima</i> <i>Phormidium ectocarpi</i>	C-PE; C-PC; AlloPC. C-PC; Allo PC C-PE	Hattori and Fujita (1959) O'hEocha (1958) O'hEocha (1955, 1960).
<u>CRYPTOPHYTA</u>		
<i>Hemiselmis virescens</i> <i>Cryptomonas ovata</i> <i>Sennia sp.</i> <i>Cyanidium caldarum.</i>	PC. PE. PE; PC C-PC.	Allen et al. (1959). Haxo and Fork (1959). O'hEocha and Raftery (1959). Allen (1959).

because of their lability, although there are some reports of local usage by communities living by the sea. Newton (1951) reported that the Red alga *Ceramium Rubrum* was once used as a source of rouge by Hebridean girls.

Nomenclature

The term "bilichromoprotein" was proposed by Haxo et al. (1955) as a general name for these proteins, and this name was modified to "biliprotein" by O'hEocha (1958); the prefix "bili-" indicates the relationship between the prosthetic groups of the proteins and the "bile pigments". (Lemberg and Legge, 1949). These prosthetic groups will be discussed at greater length further on. Other terms that have been used, include "phycochromoproteids" (Kylin, 1937); "tetrapyranyl proteins", (Haurowitz, 1958), and "phycobiliproteins" (Bogorad, 1965).

The biliproteins are either red in colour (phycoerythrins) or blue (phycocyanins), and are characterised by the brilliant fluorescence of their aqueous solutions - red in the case of the phycoerythrins, and blue for the phycocyanins.

The visible spectra of the phycoerythrins may have one, two, or three maxima - the prefixes C-, B-, and R- respectively are used to distinguish these three types. Originally these letters were selected to designate phycoerythrins occurring respectively in the Cyanophyta, Bangiales, and other Rhodophyta, but now the distinction is purely on the basis of spectral properties. In a similar manner, C- and R-phycocyanin, which were formerly thought to originate exclusively from the Cyanophyta and Rhodophyta respectively,

are now distinguished solely by their spectral properties. C-phycoerythrin has but one maximum in its visible absorption spectrum, and R-phycoerythrin two. A further phycoerythrin-allophycoerythrin - formerly known as P-phycoerythrin - is widely distributed in algae, and its name derives from this fact, the P being an abbreviation for "Pan".

In 1959, it was reported independently from three sources (Allen et al, 1959; Haxo and Fork, 1959; O'hEocha and Raftery, 1959) that biliproteins occur in members of the Division Cryptophyta (Cryptomonad algae). Three distinct Phycoerythrins and at least three phycoerythrins, differing in spectral properties from those types already considered, have been reported. The distribution of biliprotein types is thus not exclusive, and it is logical that prefixes should derive from the spectral properties of the protein, rather than the taxonomic position of the alga from which the particular protein is obtained.

Diagrams 1 and 2 show typical phycoerythrin and phycoerythrin absorption curves; in Table 2 are listed the absorption maxima of various biliproteins.

Biological Function of Biliproteins

Although photosynthesis in algae and higher plants seems to be limited to those forms which contain chlorophyll a, it has long been recognised that other pigments may contribute to the photosynthetic process. This fact was first reported by Engelmann (1883, 1884); from his experiments, which involved the use of motile aerotactic bacteria as oxygen indicators, he concluded that the phycobilins of Red and Blue-

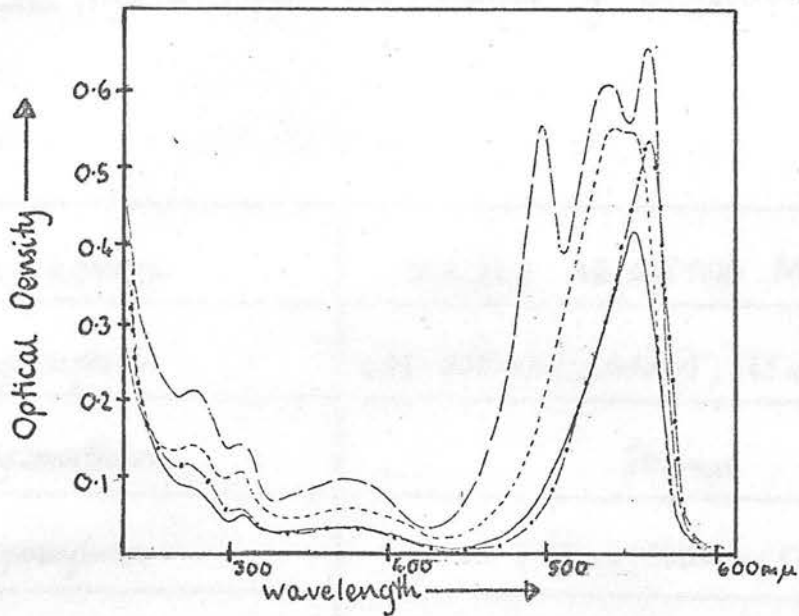


DIAGRAM 1 Typical Phycoerythrin Absorption Curves.

- R-Phycoerythrin [*Ceramium Rubrum*].
- B-Phycoerythrin [*Porphyridium Cruentum*].
- · - · - · C-Phycoerythrin [*Phormidium Persicinum*].
- Cryptomonad Phycoerythrin [*Hemiselms Rufescens*].

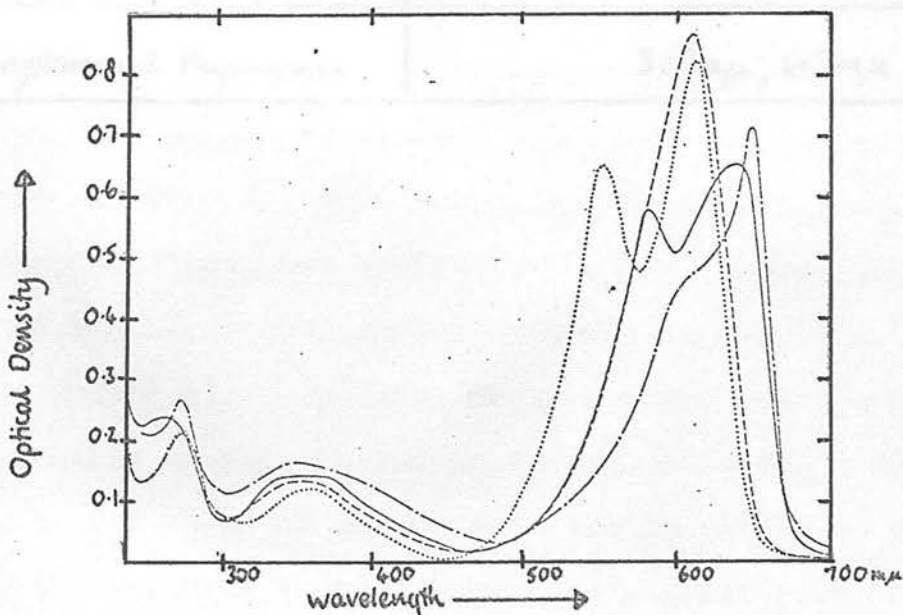


DIAGRAM 2 Typical Phycocyanin Absorption Curves.

- R-Phycocyanin [*Porphyra Laciniata*].
- C-Phycocyanin [*Nostoc Muscorum*].
- · - · - · Allophycocyanin [*Nostoc Muscorum*].
- Cryptomonad Phycocyanin [*Hemiselms Virescens Droop*].

TABLE 2. Typical Absorption Maxima of Biliproteins.

BILIPROTEIN	VISIBLE ABSORPTION MAXIMA.
B-Phycocyanthrin	495-500m μ (shoulder); 545m μ ; 565m μ
C-Phycocyanthrin	565m μ
R-Phycocyanthrin	495m μ ; 540m μ ; 565m μ
C-Phycocyanin	615m μ .
R-Phycocyanin	553m μ ; 615m μ .
Allophycocyanin	620m μ (shoulder); 650m μ .
Cryptomonad Phycocyanthrin	556m μ .
Cryptomonad Phycocyanin	588m μ ; 615m μ .

green algae were as effective as chlorophyll a in sensitizing photosynthesis. Later work by Emerson and Lewis (1942) using the Blue-green alga *Chroococcus turgidus*, confirmed Engelmann's proposals by demonstrating that the yield of photosynthesis in the yellow region, where more than half the absorption is attributable to phycocyanin, is as high as that of chlorophyll-sensitized photosynthesis in the Green alga *Chlorella pyrenoidosa*.

The experiments mentioned above suggest that energy absorbed by pigments other than chlorophyll can be utilised for photosynthesis. It was not until 1950 that it was suggested (Arnold and Oppenheimer, 1950) that transfer of energy between dissimilar molecules (from phycobilins to chlorophyll) provided a probable explanation of the high yield of photosynthesis in *Chroococcus* in the spectral region where most of the energy is absorbed by phycocyanin. It is worthy of note that the concept of energy transfer in photosynthesis was not a new one, although Gaffron and Wohn (1936) who had applied the concept to explain the results of experiments by Emerson and Arnold (1932) on the kinetics of photosynthesis in flashing light, had applied it only to energy transfer between similar (chlorophyll) molecules. In 1943, Dutton et al. showed directly that energy absorbed by one pigment can actually be transferred in vivo to another. Their results, and those of Dutton and Manning (1941) on sensitized fluorescence, thus support Engelmann's contention that the accessory pigments act only as light absorbers, and are not themselves photocatalysts.

Haxo and Blinks (1950), using polarographic measurements of oxygen evolution, determined action spectra of photosynthesis in a number of different algae, and compared these with the absorption spectra of intact thalli in each case. From their results, they concluded that in Red algae, chlorophyll plays only a minor role in photosynthesis, the phycobilins being the pigments of major importance. They reported only weak chlorophyll activity in the Blue-green algae *Anabaena* and *Oscillatoria*, in contrast to the results of Emerson and Lewis (1942) on *Chroococcus*. Duysens (1952), using a technique similar to that of Haxo and Blinks, measured photosynthesis in the Red Alga *Porphyridium cruentum* and obtained similar results. Although he confirmed the low yield of chlorophyll sensitized photosynthesis, Duysens still considered chlorophyll to be the primary photocatalyst, since results obtained by other workers (Van Norman et al., 1948; French and Young, 1952; also Duysens, 1952) had shown that energy absorbed by phycoerythrin strongly sensitized chlorophyll fluorescence.

These effects are of considerable ecological importance in coastal sea-water, where daylight is attenuated rapidly, and green radiation penetrates more deeply than blue or red. This is due to selective absorption and scattering of light by the water plankton and suspended matter, and the absorption of blue radiation by the "yellow substance" found in these waters. The chlorophylls transmit green light, but phycoerythrin, the amount of which increases with the depth of growth of Red Algae, is an effective absorber of green radiation, and the energy which it absorbs thus it transfers

to chlorophyll a. The biliproteins are also effective accessory pigments in the enhancement effect - the light they absorb is essential for the efficient photosynthetic utilisation of the light energy absorbed by the chlorophyll a itself.

Factors influencing occurrence of biliproteins

The biliprotein content of Red and Blue-green algae is influenced by various factors. These factors include

- (1) Intensity and nature of illumination
- (2) Temperature
- (3) Composition of the culture medium
- (4) The season of the year

A biliprotein content of 2% for a marine Red alga is about the highest reported value; a value of 24% of phycocyanin (dry weight) was reported in *Anacystis Nidulans* grown at 39°C and at low light intensity (Myers and Kratz, 1955). At low light intensity, green light was found to be more effective than blue light in stimulating the formation of phyco-erythrin (Brody and Emerson, 1959). Halldal (1958) reported that *Anabaena* sp., which forms only phycocyanin at a high light intensity, forms phycoerythrin in addition at lower intensities. On the other hand, *Anacystis Nidulans* does not form phycoerythrin under any light condition. Hattori and Fujita (1959a) reported that the ratio of phycoerythrin to phycocyanin in *Tolypothrix Tenuis* depends on whether the alga was grown in fluorescent or incandescent light. In a chlorophyll-less mutant of *Cyanidium caldarium*, Nichols and Bogorad (1960) found that

phycocyanin is formed in light of either 450 or 600 m μ , but not in light of 550, 660, or 700 m μ . Complementary chromatic adaptation (the enhanced formation of the pigment that most strongly absorbs the incident light) is completely effective only at low light intensity. Study of the reactions leading to biliprotein synthesis requires that both intensity and colour of the illumination be varied.

The effect of temperature on the turnover of Phycocyanin in *Oscillatoria Subbrevis* has been studied by Garnier (1962).

Phosphorus is one of the major nutrient elements required for normal algal growth, but there is no evidence to suggest that phosphorus deficiencies or excesses influence the relative amounts of the biliproteins in algae. Sodium or molybdenum deficiencies in Blue-green algae have been found to correlate with a decrease in the amount of phycocyanin formed (Fogg, 1952; Allen and Amon, 1955); an iron deficiency reduces the concentrations of both phycocyanin and phycoerythrin (Boresch, 1921). Calcium is required in macroquantities by Blue-green algae, but only in microquantities by Red algae. Various other trace metals - notably manganese, vanadium, cobalt, zinc, copper and boron, are required, but no correlations linking these metals with the relative amounts of the biliproteins have been obtained. A notable feature of algal requirements is the absence of a need for potassium. The importance of the presence of these various trace elements is indicated by a two-hundred-fold increase in the growth rate of *Anabaena cylindrica* when the concentrations of the various micronutrients were at optimum values.

Culture media deficient in nitrogen limit the formation

of biliproteins (Fogg, 1952). Pre-illuminated nitrogen deficient cultures of *Tolypothrix* formed biliproteins in the dark when nitrate was added to the medium (Hattori and Fujita, 1959). The ratio of the pigments formed was affected by the character of the light used during the pre-illumination period: green light favoured the synthesis of phycoerythrin, red light, that of phycocyanin. In cultures grown heterotrophically in darkness, phycocyanin was still formed, but phycoerythrin was not (Hattori and Fujita, 1959; Fujita and Hattori, 1960).

The biliprotein content was shown by Lemberg (1928) to vary with the season. It accounted for about 1.9 per cent (dry weight) of *Ceramium rubrum* in December to February, but dropped to about half this value in March. On the other hand, this species contained relatively more phycocyanin in March than in December.

Isolation and Purification of the Biliproteins

Rupture of the cell-walls of algae containing biliproteins leads to release of the biliproteins into the surrounding medium. Various mechanical methods have been used to rupture the cell walls - these include maceration, grinding with an abrasive agent such as sand or glass wool, ultrasonic oscillation, and repeated freezing and thawing. Centrifugation of the aqueous extract then yields a crude biliprotein solution.

Fractionation of the crude biliprotein solution and the removal of other water-soluble constituents of the algal cells, was formerly brought about by fractional precipitation using

ammonium sulphate; this, however, is a slow and tedious operation, particularly for those proteins which are present in low concentrations. A more satisfactory procedure is adsorption chromatography of the crude biliprotein solution on a column of tricalcium phosphate gel; this method was originally used by Swingle and Tiselius (1961), and has since been used for the purification of biliproteins from many sources (Haxo and Blinks, 1950; Jones and Blinks, 1957; and O'hEocha and Haxo, 1960). The biliproteins are eluted with phosphate buffers of increasing concentrations, and it is generally found that the phycoerythrins are eluted before the phycocyanins. Direct adsorption on tricalcium phosphate has been used for the large scale preparation of biliproteins (Tiselius, 1954).

Gel filtration is another technique used to resolve biliprotein mixtures. In a typical experiment, Nultsch (1962) applied the protein mixture obtained from *Phormidium autumnale* to a Sephadex column, and found that the phycocyanin migrated faster than the phycoerythrin. Sephadex gel filtration is now widely used in the purification of biliproteins, either as a means of removing low molecular weight contaminants or for desalting purposes (Raftery and O'hEocha, 1965; Eriksson and Halldal, 1965).

Phycocyanin and phycoerythrin were completely separated by Zone Electrophoresis (Hjerten, 1958), in a horizontal, rotating polythene tube, which allowed separation of the substances in a medium of relatively constant composition. Hjerten purified the separated biliproteins by "molecular sieving" on polyacrylamide gels (Hjerten, 1963). With Mosbach

(Hjerten and Mosbach, 1962) he also separated the above biliproteins using Disc Electrophoresis, a technique which will be discussed at a later stage in the practical section.

Eriksson and Halldal (1965) used Diethyl aminoethyl (DEAE) cellulose to purify the biliproteins from the Red algae *Ceramium rubrum*, *Porphyra lineans* and *Porphyra umbilicalis* f. *laciniata*, and claimed that it was a convenient, fast and relatively simple method.

Ion-exchange resins have been successfully used for the chromatography of biliproteins; Boman and Westlund (1956) purified phycoerythrin and phycocyanin using the strong anion exchange resin, Dowex-2 (Cl^-).

Fujiwara (1955) suggested the use of Rivanol (2-ethoxy-6,9-diamino-acridine lactate) to precipitate the biliproteins from *Porphyra tenera* and separate them from mucous substances. Difficulty has been experienced since, however, in removing the last traces of rivanol from the preparations thus obtained.

Crystallisation of the Biliproteins

Successful crystallisation of the biliproteins was first carried out in 1928 by Svedberg and Lewis, who obtained crystals of phycocyanin and phycoerythrin from *Ceramium rubrum*, by the carefully controlled addition of ammonium sulphate. The first photographs of biliprotein crystals were published by Lemberg (1930), and more recently by Fujiwara (1955), who reported that R-phycoerythrin formed either needles or prisms, while C-phycocyanin formed either needles or platelets. Hattori and Fujita (1959a) used a combination of the direct adsorption method and fractional precipitation to obtain

crystalline preparations of a number of biliproteins.

Airth and Blinks (1957) made the surprising discovery that fresh preparations of B-phycoerythrin from *Porphyra naiadum* could not be crystallised by ammonium sulphate precipitation, but if the protein solutions were allowed to "age" at room temperature, then crystallisation readily occurred. This suggests that some spontaneous change occurs in the phycoerythrin molecule on standing.

In 1964, Leibo and Jones obtained orthorhombic crystals of B-phycoerythrin from *Porphyridium cruentum* and also estimated that one cell of that alga contained approximately 6.10^{-14} grams of phycoerythrin.

O'Carra (1965), using ammonium sulphate fractionation, crystallised R-phyococyanin and R-phycoerythrin from *Porphyra laciniata*, and B-phycoerythrin from *Rhodochorton floridulum*. O'hEocha and Raftery (1959) isolated and purified R-phycoerythrin from *Ceramium rubrum* in a similar manner, and used starch-gel electrophoresis as an additional purification procedure. When the ratio of the extinction coefficient of the main peak in the visible region to that of the protein peak at 278 m μ was greater than 4, the protein was considered to be highly purified. This provides a useful, if somewhat arbitrary, criterion of biliprotein purity.

Physical properties of the biliproteins.

The spectral properties of the biliproteins have already been mentioned; phycocyanins have one or two absorption maxima in the visible spectrum, while the phycoerythrins have

one, two or three such maxima. These spectra can be explained in terms of the prosthetic groups and their environment, and this matter will be discussed at length at a later stage. In the ultra-violet region, both phycocyanins and phycoerythrins show maxima at 270-280m μ , this being due to the presence of tyrosine and phenylalanine residues; phycocyanins show a lesser, broad peak at about 350 m μ , and a shoulder at 285 m μ due probably to tryptophane residues has also been reported (Hattori and Fujita, 1959a), while phycoerythrins have a maximum at around 305-310 m μ .

One of the most striking properties of aqueous bili-protein solutions is their fluorescence under visible irradiation. Phycocyanin solutions, which are blue in colour, emit red fluorescence, which is reported, for C-phycocyanin, to be most intense at pH 6 to 6.5 (Lavorel and Moniot, 1962). The fluorescence maximum has been reported at various wavelengths between 637 and 680 m μ (Berns, Crespi and Katz, 1963). All phycoerythrin solutions, which are red in colour, emit orange fluorescence, with the maximum at 578 to 580 m μ (French et al, 1956). The fluorescence of R-phycoerythrin is stated to be most intense at pH 7.5 (Krasnovskii et al., 1952).

The mean value of the molecular weight of C-phycocyanin near its iso-electric point (pH 4.7) was given as 208,000 \pm 5,000, by Svedberg and Katsurai (1929), who also reported that one-third of the biliprotein existed as half-molecules at pH 6.8, and that at pH 12 it existed as units of one sixth of the original size. Later, in the same laboratory, Eriksson-Quensel (1938), found that the molecular weights quoted by

the earlier workers for R-phycoerythrin and R-phyococyanin (both $208,000 \pm 5,000$) were low because the sedimentation constants which were used had not been corrected for the density and viscosity of the solvent. Although she did not re-examine the molecular weight of C-phyococyanin, it is a reasonable assumption that its true molecular weight is higher, in the region 270,000 to 290,000. Hattori and Fujita (1959) found the molecular weight of C-phyococyanin to be 138,000, but this value was determined at pH 7.2, and it is probable that the value nearer the iso-electric point would be double this, i.e. 276,000, a view which is supported by sedimentation constant measurements. Disaggregation of C-phyococyanin from *Plectonema calothricoides* is observable even at pH 4.7 (Berns et al., 1963), and appreciable amounts of low molecular weight components were detected when C-phyococyanin was analysed centrifugally in the presence of urea and sodium dodecyl sulphate. On the basis of results thus obtained and of diffusion coefficients determined from immunodiffusion experiments, the minimum molecular weight of C-phyococyanin was reported to be 30,000 (Berns et al., 1964).

Bergeron (1963) found that the absorption and fluorescence spectra of C-phyococyanin from *Anacystis nidulans* were pH dependent. The spectrum at pH 5.7 was taken to represent C-phyococyanin dimer (M.W.c. 276,000) and that at pH 7.5 the monomer (M.W.c. 138,000). The implication of Bergeron's work is that C-phyococyanin is partly aggregated in vivo.

Interesting results were obtained by Berns and Edwards (1965) who investigated C-phyococyanin from *Plectonema*

calothricoides using the electron microscope and the contrasting technique. Potassium phosphotungstate and uranyl acetate were used as contrast agents. Round structures with a central hole were evident, and were interpreted as being the hexamer structure postulated from independent physical studies. Higher magnification demonstrated the hexamer to consist indeed of six globular monomer units, arrayed approximately at the vertices of a regular hexagon.

Recent investigations on the association-dissociation reactions of C-phycocyanin have not only confirmed the dependence on pH, but have also shown a dependence on ionic strength, protein concentration, and temperature. (Hattori et al., 1965). Scott and Berns (1965), from studies of sedimentation velocity as a function of pH, ionic strength, temperature, and buffer, provided further evidence in support of the ring-like hexamer concept, and they also postulated a monomer-trimer-hexamer-dodecamer equilibrium.

Both Hattori et al. (1965) and Scott and Berns (1965) made extensive use of deuteriophycocyanin-phycocyanin with hydrogen completely replaced by deuterium - in their investigations. Such deuteration - carried out by culturing algae in heavy water - results in a shift of ca. 7 m μ of the visible absorption maxima, irrespective of the absorption state.

Phycocyanin molecules are negatively charged in those pH regions where association-dissociation reactions occur. An increase in the hydrogen-ion concentration will therefore lead to a reduction of the net charge on the molecule, and

hence to the formation of monomers. Increasing the ionic strength has a similar effect, by reducing the electrostatic interactions. However, if the association of monomers were regulated solely by electrostatic forces, then the association-dissociation reactions of deuteriophycocyanin and ordinary phycocyanin would be expected to be of similar orders, and in fact the associating tendency of deuteriophycocyanin is several times less than that of ordinary phycocyanin. A reasonable explanation is that hydrophobic side chains participate in an important and specific way in the union of sub-units, and that deuteration reduces the extent of interaction between hydrophobic side-chains of individual monomers.

Berns and Scott (1965) studied a thermophilic phycocyanin from *Synechococcus lividus*, and found that at 50°C - the culture temperature of the alga - the distribution of aggregating species was almost identical with that of normal phycocyanin at 25°C, and they suggested that this phenomenon was caused by a greater number of charged and polar residues in this particular protein. Their findings support the opinion that the hexamer and dodecamer are of importance in vivo, and suggest a mechanism by which aggregating proteins from thermophilic organisms may adapt to the "proper" aggregation equilibrium at higher temperatures.

An alternative method of determining the molecular weight of phycocyanin uses the results of amino-acid analyses, and this will be discussed fully in that section.

R-phycocyanin was found by O'Carra (quoted in O'hEocha, 1965) to give two fluorescence maxima, at 565 and 637 mμ. The finding of C-phycocyanin in a number of Rhodophyta over the

past few years has led to the claim that R-phycoerythrin is in fact C-phycoerythrin contaminated with phycoerythrin (Hattori and Fujita, 1959). However the use of such fractionation techniques as counter-current distribution (Albertson and Nyns, 1959), DEAE-cellulose chromatography, and starch-gel electrophoresis (O'Carra and O'hEocha, 1965), has shown that R-phycoerythrin behaves as a homogeneous protein. The absorbancy values at its two maxima are similarly affected by urea denaturation, and the difference spectrum of R- and C-phycoerythrin rules out the possibility of phycoerythrin contamination (O'Carra and O'hEocha, 1959). R-phycoerythrin therefore appears to be a distinct biliprotein. R-phycoerythrin is stable in the pH range 2.5 to 6.0, with a molecular weight of about 273,000, but it dissociates to particles of about half this size at pH 7 to 8.5 (Eriksson-Quensel, 1938).

Allophycoerythrin, in spite of its name ("allo" meaning different from normal) is now accepted as an *in vivo* biliprotein. It has been crystallised as thin platelets, and its molecular weight, estimated by ultra-centrifugation, is 134,000 at pH 7.2. It dissociates irreversibly at pH 11.6 (Hattori and Fujita, 1959). The fluorescence maximum of allophycoerythrin is at 663 mμ.

Airth and Blinks (1956) found the molecular weight of B-phycoerythrin to be around 290,000; a slightly lower value has been reported for B-phycoerythrin from *Porphyridium cruentum* (Brody and Brody, 1961). The various absorption bands of B-phycoerythrin - at 500, 545 and 656 mμ in the case of B-phycoerythrin from *ceramium rubrum* - have recently been shown to be independent of one another. The 500 mμ and

545 m μ bands disappear on reduction with sodium hydrosulphite (Jones and Fujimori, 1961); O'hEocha and O'Carra (1961) showed that the 500 m μ band behaved independently of the other two; and the 565 m μ band disappears on the addition of p-chloro-mercuribenzoate, a sulphydryl blocking agent (Jones and Fujimori, 1961; Fujimori and Quinlan, 1963). Fujimori (1964) showed that phycoerythrin could be split into two units each containing different chromophores, and was able to separate a modified component of phycoerythrin. He also reported an interaction of this component with glutathione, suggesting possibly that the chromophore becomes inactive on treatment with p-chloro-mercuribenzoate, conceivably through the fission of a sulphur-sulphur linkage. This work was carried on by Fujimori and Pecci (1967), who investigated the action of various mercurials on the intact protein and demonstrated the existence of several sub-units from which the protein is presumably assembled. No dissociation in untreated samples of the protein was described, and the protein was reported to show only one component on gel filtration. However, in 1967 Nolan and O'hEocha reported the separation of a lower molecular weight component from R-phycoerythrin on gel filtration, and in 1968 Mieras and Wall showed that B-phycoerythrin did in fact dissociate, giving two low molecular weight components on Sephadex filtration. They postulated that B-phycoerythrin may in fact follow the monomer-trimer-hexamer-dodecamer pattern observed in the C-phyococyanin system and which has already been discussed.

C-phycoerythrin has been reported to have a molecular weight of 226,000 in the pH range 5.2 to 7.2. At pH 8.3, the

molecule begins to dissociate into smaller units (Hattori and Fujita, 1959).

The molecular weight of R-phycoerythrin was reported by Eriksson-Quensel (1938) to be about 291,000, and this value was reported to hold between pH 3 and 10. R-phycoerythrin samples from fourteen species of Red algae were found to give a molecular weight of 290,000 (Nolan and O'hEocha, 1967). The same workers isolated forms of R-phycoerythrin of molecular weights around 48,000, thus supporting qualitatively the results of Hjerten (1963), who showed that R-phycoerythrin gave two zones on polyacrylamide gel electrophoresis and on ultracentrifugation.

Nolan and O'hEocha (1967) carried out molecular weight determinations on cryptomonad biliproteins and found that their molecular weights were much less than those of biliproteins from other algal divisions. A typical cryptomonad phycoerythrin gave a molecular weight of 27,800, and a typical phycocyanin, 37,300.

Immunological Studies

Berns (1963) presented immunological evidence which supports the conclusion that the unassociated deuterio- and protio-phycocyanins from *Plectonema calothricoides* are identical in those structural aspects to which the techniques used are sensitive. The observed differences between the two proteins must therefore be considered purely in terms of a difference in isotopic substitution.

Berns (1967) showed that C-phycocyanin from all sources were antigenically and immunogenically related, and apparently

also related to allophycocyanin, but not to any of the phycoerythrins. He discovered larger antigenic differences among phycoerythrins from different groups of algae, and characterized the role of aggregation of the individual biliproteins in their immunochemistry. The immunochemical aspects of the biliproteins were striking in that protein antigens from vastly different cell types were found to be closely related - a relationship which may be interpreted as suggesting that Rhodophyta evolved from Cyanophyta or from some common ancestral stock.

Analyses of Biliproteins

Elementary Composition. Crystalline samples of C-phycoerythrin, C-phycocyanin, and allophycocyanin, all obtained from *Tolypothrix Tenuis*, were all found to give similar elementary analyses, i.e., about 49 per cent C, 7.5 per cent H, and 15 per cent N. The two phycocyanins were reported to contain 0.65 per cent S, and no ash, while C-phycoerythrin had 0.10 per cent S, and 0.37 per cent ash. (Hattori and Fujita, 1959). Higher values - about 1.6 per cent - have been reported elsewhere (Akabori and Fujiwara, 1958; Raftery and O'Heocha, 1965) for the sulphur content of R-phycoerythrin and R-phycocyanin. The latter workers managed to recover about two-thirds of the sulphur content of R-phycoerythrin from *ceramium rubrum* as sulphur-containing amino-acids; they pointed out that ammonium sulphate was not used during the purification of the protein. Kimmel and Smith (1958) also failed to account for the total sulphur content of R-phycoerythrin and C-phycocyanin from *Porphyra*

tenera, but complete recovery of elementary sulphur, as sulphur-containing amino-acids, was reported from C-phyco-cyanin from *Nostoc Muscorum* (Raftery and O'hEocha, 1965).

Glendenning (1954) obtained a 4 per cent yield of the tetrapyrrole chromophore group from C-phyco-cyanin, a figure which indicates about sixteen chromophore groups per molecule. Brody and Brody (1961) used a non-destructive assay method based on the particle weight, specific extinction coefficient, fluorescence lifetime, and fluorescence yield. They, too, concluded that there were sixteen chromophore groups per phyco-cyanin molecular weight unit of 273,000. If the chromophore group has a molecular weight of approximately 590, then the above figures indicate that the protein contains 3.4 per cent chromophore by weight. The latter authors suggest that while different biliproteins may be capable of binding different numbers of chromophore groups, it is also possible that it is the availability of the chromophore in the cell that determines the number of pigment groups per biliprotein molecule.

It has generally been found that the yields of amino-acids obtained from biliproteins do not account for the total mass of the protein; this discrepancy is partly explained by the chromophore content, which has already been mentioned, and also by the fact that the biliproteins are found to contain varying amounts of carbohydrate. The observation that R-phycoerythrin is a glycoprotein was made by Fujiwara (1961), who reported the occurrence of 4.78 per cent carbohydrate in R-phycoerythrin from *Porphyra tenera*. This was later

confirmed by Raftery (1965), working with purified R-phycoerythrin from *Ceramium rubrum*. Carbohydrates have also been identified in some of the chromopeptide material obtained from peptic digests of R-phycoerythrin (Fujiwara, 1960). The presence of carbohydrate in C-phyococyanin from *Porphyra tenera* was reported by Fujiwara (1961); Sasaki and Tsuchiya (1961) reported that C-phyococyanin from the same source yielded, on hydrolysis, at least seven sugars, the most important being xylose, mannose, glucose, and galactose. An interesting possibility which arises is that, if the carbohydrate were present as a sulphate ester, as is the case with certain marine algae, this could explain the fact - already mentioned - that many workers have failed to account for the total sulphur content of their biliprotein preparations in terms of sulphur-containing amino-acids.

Amino-acid analyses. Amino-acid residues have been reported to account for between 75 and 89 per cent of the total biliprotein weight, although an exceptional amino-acid recovery of 102 per cent was reported from one investigation of C-phyococyanin (Kimmel and Smith, 1958). Results for a number of biliproteins from various sources are shown in table 3.

The general picture to emerge from these analyses is that algal biliproteins are richer in dicarboxylic (acidic) than in basic amino-acids, and that they have a high content of amino-acids having hydrophobic side-chains. An estimate of the iso-electric point of C-phyococyanin from its amino-acid composition, showed good agreement with that reported from electrophoretic studies - i.e. pH 4.76 (Kimmel and Smith, 1958).

TABLE 3a; Amino Acid Compositions of Various Biliproteins (Raftery and O'Neocha, 1965).

[Percentages by weight of recovered amino acids].

	R-Phycoerythrin [Rhodysmenia Palmata.]	B-Phycoerythrin [Porphyridium Cruentum.]	C-Phycoerythrin [Phormidium Persicinum.]	R-Phycocyanin [Rhodysmenia Palmata.]	Allo-Phycocyanin [Arthrospira Maxima.]
Alanine	11.3	11.9	15.3	16.6	9.6
Arginine	3.7	7.0	6.6	3.5	4.0
Aspartic Acid.	10.7	11.1	12.5	11.2	9.2
Cystine	0.8	0.5	0.5	0.5	1.5
Glutamic Acid.	9.4	8.9	10.3	13.5	14.0
Glycine	5.5	10.0	8.6	4.8	6.0
Histidine	1.7	2.0	1.8	1.5	1.6
Iso-leucine	4.1	4.9	5.0	3.9	3.7
Leucine	11.1	7.2	8.5	10.4	9.7
Lysine	3.9	6.2	4.5	6.0	2.6
Methionine	2.2	1.0	1.0	1.3	2.2
Phenylalanine	4.0	4.0	3.2	1.6	2.3
Proline	5.4	4.3	2.3	1.0	4.9
Serine	7.1	7.9	8.0	9.0	6.1
Threonine	5.1	5.8	3.5	6.1	9.3
Tyrosine	4.1	3.7	2.0	3.6	5.4
Valine	9.9	7.1	9.0	5.5	7.9

TABLE 3b; Amino Acid Compositions of Various C-Phycocyanins (① O'Reilly and Berns, 1963; ② present work).
[Numbers of residues per whole cystine].

	① Plectonema Calothricoides.	① Synechococcus Lividus. [thermophilic]	① Phormidium Luridum.	① Porphyra Tenara.	① Nostoc Muscorum	② Anabaena Cylindrica.
Alanine	40	26	43	19	41	38
Arginine	14	19	17	7	18	15
Aspartic Acid	28	28	29	14	32	27
Cystine [as Cysteic Acid].	1	1	1	1	1	1
Glutamic Acid	18	30	19	13	29	25
Glycine	20	10	24	11	23	25
Histidine	2	2	2	1	2	2
Isoleucine	14	16	18	7	14	13
Leucine	22	23	25	13	25	25
Lysine	10	11	13	4	11	12
Methionine.	8	8	9	4	2	3
Phenylalanine	8	10	11	3	7	9
Proline.	8	10	9	5	10	10
Serine.	16	10	20	12	22	21
Threonine.	14	14	17	7	18	19
Tyrosine	10	16	14	7	12	11
Valine.	16	17	20	9	15	21

A calculation based on the content of amino-acid and ammonia residues of R-phycoerythrin from *Ceramium rubrum* indicated an isoelectric point in reasonable agreement with that established by electrophoresis - i.e. pH 4.3 (Raftery and O'hEocha, 1965). A similar calculation by Kimmel and Smith (1958) using R-phycoerythrin from *Porphyra tenera*, did not, however, give a satisfactory result.

As has already been mentioned, amino acid analyses afford a further means for the estimation of the molecular weights of biliproteins. Berns, Scott and O'Reilly (1964) found that the minimum molecular weight of C-phyococyanin from a number of algal sources was around 30,000; the calculation was based on the amounts of the least abundant amino-acids, lysine and histidine. This value agrees with that determined ultracentrifugally by Berns et al. (1964). The minimal molecular weight of C-phyococyanin from the Red alga *Porphyra tenera* was calculated as 38,100 (Kimmel and Smith, 1958). O'Carra (1965) estimated the molecular weight of C-phyococyanin as 138,000 on the basis of N-terminal analyses.

The only available analysis of C-phycoerythrin (Raftery and O'hEocha, 1965) indicates a minimum molecular weight of about 61,500, assuming one cystine residue per integral unit. However, since both cystine and cysteine tend to give low yields on direct hydrolysis of proteins, the minimum molecular weight of C-phycoerythrin thus estimated is likely to be high. In B-phycoerythrin from *Porphyridium cruentum*, a total of eight sulphydryl groups per molecular weight unit of 290,000

has been estimated by amperimetric titration (Fujimori and Quinlan, 1963).

Terminal Groups. Qualitative N-terminal analyses on R-, B-, and C-phycoerythrins showed that methionine was the sole N-terminal amino-acid residue in each case (O'Carra and O'hEocha, 1962). Quantitative N-terminal analyses have been reported for R-phycoerythrin from *Ceramium rubrum*; in one analysis by the Edman method nine methionine residues were obtained per molecular weight unit of 290,000; a small quantity of N-terminal aspartic acid was also observed (Vaughan, 1963). Using the Sanger method, O'Carra (1965) found fourteen N-terminal methionine residues per molecular weight unit of 290,000 in the same protein, a value in better agreement with available C-terminal and total amino-acid analyses.

C-phycoerythrin from *Nostoc muscorum* has been reported to contain two N-terminal threonine and four C-terminal serine residues per molecular weight unit of 276,000 (O'Carra, 1965; O'hEocha and Raftery, 1959). Results from amino acid analyses and sedimentation and immunodiffusion experiments indicate a minimum molecular weight for C-phycoerythrin of about 30,000, a value which is substantially lower than that indicated by either of these terminal group analyses. A possible explanation is that C-phycoerythrin contains masked or non-amino-acid terminal groups, or - possibly - is cyclic.

Alanine was found to be the only C-terminal amino-acid residue in R-phycoerythrin from *Ceramium rubrum*, to the extent of twelve residues per unit of molecular weight

290,000 (Raftery and O'hEocha, 1965). The values of the minimal molecular weight of R-phycoerythrin calculated from the total amino-acid and C- and N-terminal analyses agree reasonably well, and the biliprotein appears to contain about fourteen subunits. This conclusion hinges on the assumption that all the subunits are the same: in fact, Vaughan (1963) has reported that this biliprotein may in fact consist of two different subunits.

O'Carra (1965) identified methionine and threonine as the N-terminal amino-acid residues of R-phyococyanin, and these results suggest that R-phyococyanin contains two different types of subunit, one related to the phycoerythrins, the other to C-phyococyanin.

The Phycobilins

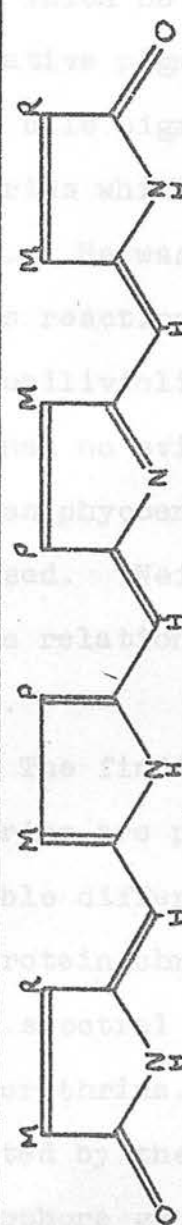
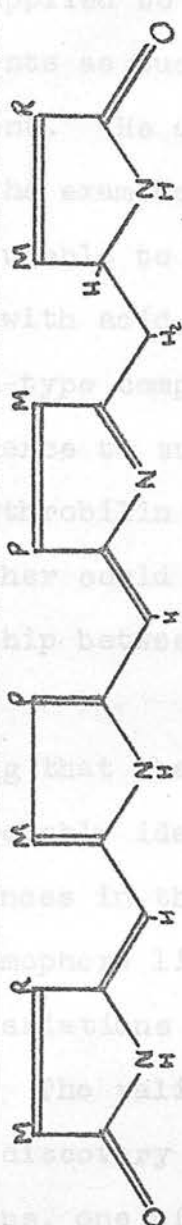
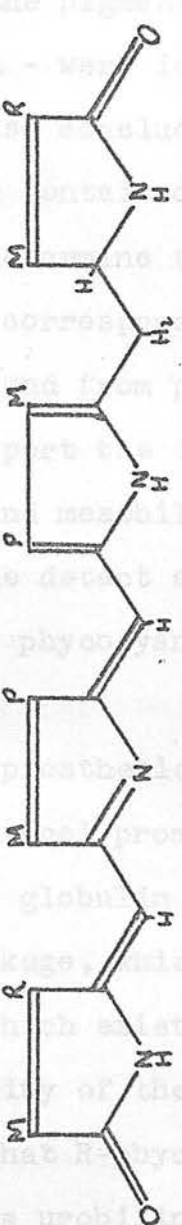
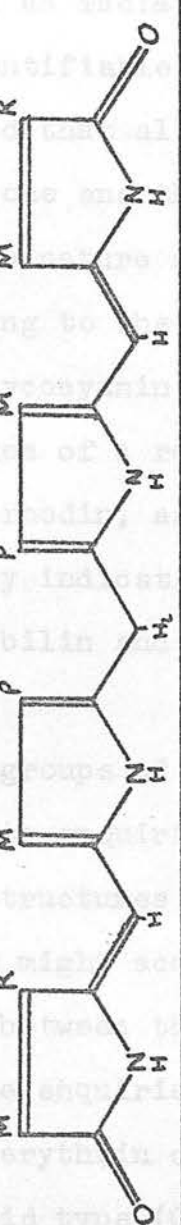
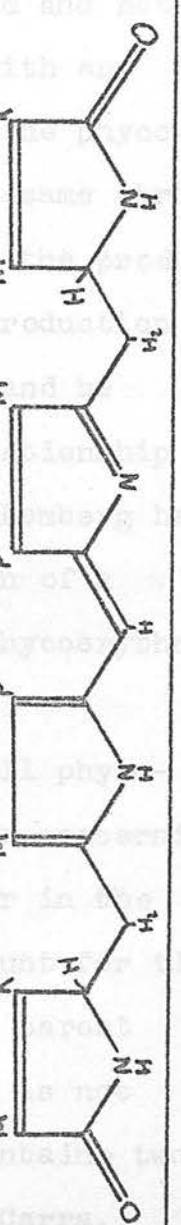
Mention has already been made of the relationship between the bile pigments and the chromophore groups of the biliproteins - the relationship which in fact led to the name biliprotein. In 1930, Lemberg showed that the biliprotein chromophore groups, now commonly referred to as phycobilins, were similar in structure to the bile pigments; the chemistry of these linear tetrapyrroles has been extensively reviewed by Lemberg and Legge (1930), and more recently by Gray (1953) and Stevens (1959).

The exact structures of the native phycobilins are still not known with certainty. The procedure employed for the isolation of a phycobilin must be selected with caution if the formation of artifact pigments is to be avoided.

In 1930, Lemberg differentiated between phycocyanobilin and phycoerythrobilin; the names refer specifically to what Lemberg considered to be the native prosthetic groups of C-phycocyanin and R-phycoerythrin respectively. The experiments of Lemberg and Bader, in 1933, led them to believe that phycocyanobilin and phycoerythrobilin, obtained from the respective chromoproteins by hydrolysis with 30 per cent methanol-HCl at 80°C, were identical with mesobiliviolin and mesobilirhodin respectively (see Fig. 1). They considered that phycocyanobilin was an oxidised form of phycoerythrobilin. However, the work of Siedel (1935) indicated that synthetic mesobiliviolin and mesobilirhodin are prototropic isomers, and Lemberg (1949) later assumed the same to be true of these particular phycobilins. It is now believed that the pigments isolated by Lemberg in his earlier work were artifacts formed during hydrolysis.

The absorption spectra of the phycocyanins and phycoerythrins can vary greatly, and this might be attributed to the existence of many different phycobilins, but spectral variations are possible also in chromoproteins containing the same prosthetic group, as is the case with the various astaxanthin-protein complexes (Fox, 1953). O'hEocha (1958) compared a number of phycobilins in an attempt to find a basis for the difference between the parent biliproteins. He used a method of hydrolysis somewhat less drastic than that of Lemberg, in order to minimise alteration of the native chromophore: dried, denatured biliprotein samples were treated with oxygen-free concentrated hydrochloric acid at room temperature, followed by dilution with water and extraction

FIGURE 1: Some Typical Bile Pigments.

NAME (Class).	STRUCTURE.
BILIVERDIN (Bilatriene); $R = -CH_2CH_3$	
MESOBILIVOLIN (Biladiene); $R = -CH=CH_2$	
MESOBILIRUBIN (Biladiene); $R = -CH=CH_2$	
MESOBILIRUBIN (Biladiene); $R = -CH=CH_2$	
i-UROBILIN. (Bilene). $R = -CH_2CH_3$	

into chloroform. On the basis of spectral evidence, he concluded that neither phycocyanobilin nor phycoerythrobilin-terms which he applied to the pigments as isolated and not to the native pigments as such - were identifiable with any known bile pigment. He also concluded that all the phycoerythrins which he examined contained one and the same chromophore. He was unable to determine the nature of the products of its reaction with acid, corresponding to the production of a mesobiliviolin-type compound from phycocyanin, and he obtained no evidence to support the idea of a relationship between phycoerythrobilin and mesobilirhodin, as Lemberg had proposed. Neither could he detect any indication of a simple relationship between phycocyanobilin and phycoerythrobilin.

The finding that the prosthetic groups of all phycoerythrins are probably identical prompts enquiries concerning possible differences in the globulin structures or in the apo-protein-chromophore linkage, which might account for the great spectral variations which exist between the parent phycoerythrins. The validity of these enquiries is not affected by the discovery that R-phycoerythrin contains two chromophore groups, one of a urobilinoid type (O'Carra, O'hEocha, and Carroll, 1964). This point will be discussed at a later stage.

Under the milder conditions which O'hEocha (1958) used to isolate these pigments, the pigment he obtained from C-phycocyanin differed from the material which Lemberg (1930) had obtained, but it was converted to a mesobiliviolin type of pigment under the conditions employed by Lemberg. O'hEocha

therefore considered that the pigment which he had obtained was in fact the prosthetic group (phycocyanobilin) of C-phycocyanin, or a close derivative. These pigments were referred to in terms of the wavelength of their respective visible absorption maxima: phycocyanobilin was referred to as phycobilin 630, and the mesobiliviolin type compound as phycobilin 608. Later, O'hEocha and Lambe (1961) isolated a third pigment, phycobilin 655, from a different algal source, and this for a time pointed to the possibility that C-phycocyanin from different plant sources might contain different prosthetic groups. However, O'hEocha (1963) studied the experimental conditions which led to the recovery of these various pigments, and concluded that the pigment which he had previously (1958) termed phycocyanobilin was in fact the prosthetic group of C-phycocyanin, regardless of its algal source. Discrepancies in the literature were attributed to slight variations in the methods of hydrolysis and purification, leading to the isolation of different pigments from C-phycocyanin.

The severity of these hydrolytic methods for the isolation of phycobilins - the least severe method required concentrated hydrochloric acid for thirty minutes at room temperature - has inevitably raised doubts as to whether the pigments thus isolated are the same as those existing in the native biliproteins. Spectrophotometric methods have been used in recent times to exclude some of the pigments obtained under such conditions from the ranks of naturally-occurring prosthetic groups; evidence has also been obtained that others of these pigments are in fact native prosthetic groups.

Rabinowitz (1951) pointed out that the spectral properties of mesobiliviolin are difficult to correlate with those of C-phyocyanin. pH difference spectrophotometry rules out the mesobiliviolinoid phycobilin 608 as the prosthetic group of C-phyocyanin, and this technique indicates that phycobilin 630 is the native pigment, phycocyanobilin. Further, phycobilin 608 and phycobilin 655 are both irreversibly formed from phycobilin 630. Also, the absorption maximum of the free base of phycobilin 630 (612 m μ in chloroform solution) is very close to that of native C-phyocyanin (615 m μ). In view of this evidence, O'hEocha (1963) applied the name phycocyanobilin to phycobilin 630.

Until recently, the only methods available for the isolation of the phycobilins were modifications of Lemberg's method, all of which involved to a greater or lesser extent, the rather drastic procedure of digestion with concentrated hydrochloric acid. In 1963, however, Fujita and Hattori showed that by refluxing phycocyanin, or even whole Blue-green algae, with methanol in the presence of ascorbic acid, the chromophore was liberated in good yield, and O'Carra and O'hEocha (1966) showed that the ascorbic acid was not in fact necessary. Crespi et al. (1967), using a variation of this method, obtained a 40 per cent yield of phycocyanobilin, assuming a 5 per cent phycobilin content, a figure which is probably rather high. They subjected this phycobilin to NMR and mass-spectral studies, and deduced a structure for it (See figure 2). They point out, however, that their distribution of the side chains is more or less arbitrary,

FIGURE 2. Pigments obtained from Biliproteins.

	STRUCTURE.
Structure for phyco-cyanobilin proposed by Cde et al., (1967) [Page 29]	
Structure for phyco-cyanobilin proposed by Crespi et al., (1967) [Page 28]	
Bilidiene IXa structure proposed for phycoerythrobilin by O'Carra et al. (1966) [Page 33]	
Protein-bound Phyco-erobilin [O'Carra et al., 1964]. [Page 34]	
"Purple pigment" obtained by methanolysis of Phyco-erythrin [Rodiger et al., 1967] [Page 35]	

and is by analogy with the known bile pigments, and also, that they had not proved that the substance which they had isolated actually occurs as such in the native protein. At the same time, Cole et al. (1967) reported the isolation of a similar material, by first denaturing the native protein with trichloroacetic acid; the denatured protein was then refluxed with methanol, and then treated with boron trifluoride in methanol, to esterify the pigment. They were able to obtain a crystalline product, and using NMR and mass spectral data, they deduced a structure for phycocyanobilin which differed only slightly from that proposed by Crespi et al. (1967) (See figure 2).

Degradative studies on phycobilins have been hampered by the difficulties involved in isolating and purifying sufficient material for classical degradation and analysis. These difficulties have been satisfactorily overcome by the chromic acid microdegradation technique, evolved by Rudiger (1967) for the study of aplysioviolins, the defence pigment of the sea hare. This technique may be used with as little as 0.1 mg. bilin and is not affected by the presence of protein, and may therefore be used for direct degradation of phycobilins while still attached to the protein. Oxidation of C-phycocyanin by this method was found to give the same bilin oxidation products - identified by thin layer chromatography - as phycobilin 630 (O'Heocha, 1958) and the "blue pigment" released by prolonged refluxing of C-phycocyanin in methanol (Rudiger et al., 1967). Both these pigments were found to have two acidic side chains, and because the phycocyanin chromophore is intermediate between the verdin and

violin types, the conjugation must extend through all four rings, with one ring incompletely conjugated. The suggested structure satisfying these conditions is shown in Fig. 2. This is the same as the structure proposed by Cole et al. (1967) for the "blue pigment", which they chose to term "phycocyanobilin", a term which should only be applied to the native prosthetic group. It remains to establish whether phycobilin 630 or the "blue pigment" is this native group; the evidence would appear to be in favour of phycobilin 630 (O'Carra and O'hEocha, 1966). The structure proposed by Chapman et al. (1967) seems less likely in view of the facile isomerization of the "blue pigment" to mesobiliverdin under alkaline conditions, and the presence of a basic pyrrolenine nitrogen in the conjugated system (O'Carra and O'hEocha, 1966).

The use of enzymic methods to cleave the chromophore from the biliprotein molecule was first investigated as long ago as 1928, by Lemberg, who obtained a coloured material, soluble in amyl alcohol, by digesting phycoerythrin with pepsin. He did not establish, however, whether this substance was in fact free of amino-acid residues, or whether it was a chromopeptide. No further enzymic work was done until 1967, when Siegelmann et al. isolated a phycobilin from C-phyco-cyanin, by first denaturing the protein with trichloroacetic acid and then digesting it with the enzyme Nagarse. The phycobilin was extracted with chloroform and esterified with boron trifluoride in methanol. After purification by thin layer chromatography, the ester was crystallised from chloroform/methanol as characteristic fibrous phycocyanobilin di-methyl ester crystals.

Chapman et al. (1968) examined the various procedures available for the cleavage of phycocyanobilin from phycocyanin. They found that the yield of the phycobilin obtained by hydrochloric acid treatment was low, and that the diacid thus produced was not particularly stable. A substantially improved yield was obtained by methanolic hydrolysis, and the diester was found to be much more stable. Variable spectral behaviour of the pigment in acid chloroform solution was found to be due to incomplete protonation, and it was found that this difficulty could be avoided by the use of methanol containing 5 per cent hydrochloric acid.

Chapman et al. (1968) confirmed the earlier results of O'hEocha (1958) who had shown that the products of hydrochloric acid cleavage were dependent on time and temperature of hydrolysis, and the acid concentration. They showed that their phycocyanobilin preparations were identical to acid-cleaved phycocyanobilin, in all probability identical to O'hEocha's phycobilin 630. They suggested the two structures for phycyanobilin suggested by Cole et al. (1967) and Crespi et al. (1967) differed simply because of individual interpretations of the experimental data, and because of variations in experimental method. They also noted that the recovery of the chromophore - even by prolonged methanolic hydrolysis - never is complete, which suggests that some of the chromophore groups are shielded. Lastly, they confirmed the finding of Lemberg and Bader (1933), that refluxing phycocyanin with a 10 per cent methanolic solution of potassium hydroxide, led to the formation of mesobiliverdin, and also prepared mesobiliverdin directly from phycocyanobilin dimethyl ester.

Lemberg (1930) showed that dilute acid quenches the fluorescence and alters the absorption spectrum of R-phycoerythrin. O'hEocha and O'Carra (1961) showed that all the phycoerythrins are denatured with loss of fluorescence by acid, and more slowly by urea. Increased chromophore activity was found to be associated with denaturation. Earlier, Lemberg (1930) reported that phycoerythrobilin, a chromophore of all three phycoerythrins, is attached to the protein both by covalent and labile bonds, and in his view it is the latter which are broken by denaturing agents, with consequent loss of fluorescence. In view of the many possible effects of urea and acid on the secondary structures of proteins, any interpretation of denaturation results of this type must be tentative. However, there is evidence that the tetrapyrrole nitrogen atoms are involved in this labile bonding. Zinc complex formation by bile pigments occurs through their nitrogen atoms; the phycoerythrobilin chromophores of denatured phycoerythrins form zinc complexes readily, while those of native phycoerythrin do not react at all. The conclusion of O'hEocha and O'Carra (1961) is that the pyrrole nitrogens of the phycoerythrobilin residues in the native phycoerythrin are masked, possibly through direct hydrogen bonding, while in the denatured protein, the pyrrole nitrogens are exposed to zinc ions by the breaking of these bonds.

O'hEocha and O'Carra (1961) showed that cryptomonad- and B-phycoerythrin both give rise to only one pigment, although the two proteins differ greatly in aqueous solution. The two maxima of the B-phycoerythrin spectrum, at 544 m μ and 566 m μ , are caused by some of the phycoerythrobilin residues

being bound in a different manner from the rest, depending presumably on the protein environment. The 544 m μ peak corresponds to that of the urea-denatured protein, and may be attributed to non-hydrogen-bonded, non-fluorescent chromophores, while the 566 m μ peak is due to hydrogen-bonded, fluorescent chromophores. The 568 and 542 m μ maxima of R-phycoerythrin may be explained in a similar way. The 497 m μ maximum of R-phycoerythrin is not shifted on denaturation, and must be attributed to a different pigment. Hydrolysis studies showed this latter to be a urobilinoid pigment. (O'hEocha, 1960). That there are two independent chromophores in R-phycoerythrin is also indicated by the fluorescence spectrum of the zinc complex of the denatured chromoprotein which exhibits maxima at 520 and 600 m μ when excited at wavelengths of 490 m μ and 540 m μ respectively.

O'Carra, O'hEocha and Carroll (1964) claimed that phycoerythrobilin isolated from phycoerythrins by the method of O'hEocha (1958) was a chemically unaltered prosthetic group of these biliproteins. They proposed a bilidiene IXa structure for it (Fig. 2). This is a structure, these authors point out, which had been previously associated by Siedel (1935) with a pigment preparation named mesobilirhodin, and by Lemberg and Bader (1933) with a pigment isolated from R-phycoerythrin. However, they showed that even if any of this compound had been formed under the conditions used by Siedel, it would have isomerised to mesobiliviolin or a urobilinoid pigment. Similarly, they showed that under the conditions used by Lemberg and Bader for the preparation of

phycoerythrobilin, the phycoerythrobilin formed is converted to a urobilinoid pigment which becomes reattached to protein fragments by means of artifact bonds, yielding urobilinoid products whose general structure is shown in Fig. 2. Such products they found to resemble the phycoerythrobilin preparation of Lemberg and Bader (1933), which probably contained a peptide chain.

O'Carra, O'hEocha and Carroll (1964) point out that phycoerythrobilin as they prepared it differs considerably in spectral properties from the native phycoerythrins, but that there is a close spectral correspondence between phycoerythrobilin and its derivatives on the one hand and denatured phycoerythrins on the other, both in the ultra-violet region of the spectrum and at wavelengths above 530 m μ . They conclude from this that the native prosthetic group has the same structure as the isolated pigment. The nature of the spectral differences between native and denatured phycoerythrins have been discussed above.

Phycoerythrobilin has the general properties of a bile pigment - its absorption spectrum lacks a soret band, and it is readily converted to typical bile pigments, e.g. to a bilene on isomerization and a bilitriene on dehydrogenation (see Fig. 1). The long wavelength absorption maxima of phycoerythrobilin and its derivatives indicate that these compounds are intermediate in spectral properties between the bilitrienes with four pyrrole rings in conjugation, and the bilenes with two, and that they may be classified with the urobilinoid bile pigments as bilidienes, with a conjugated system of three rings. However, the wavelengths of the

absorption maxima of phycoerythrobilin and its derivatives are 30 to 50 m μ less than the wavelengths of the maxima of mesobiliviolin and its derivatives, suggesting that the conjugated system of the violins is more extensive than that of phycoerythrobilin. This fact is supported by the work of Gray et al. (1961) who showed that the pigment contains one pyrrolenine nitrogen. Phycoerythrobilin is isomerized by concentrated hydrochloric acid to a mesobiliviolin and a urobilin, which differs from i- and d-urobilin in that it forms refractory covalent linkages with thiol compounds, including the apoproteins of phycoerythrins. Mesobilirhodin - a pigment obtained by the isomerization of d-urobilin - is closely related to phycoerythrobilin and is considered to be a side-chain isomer of it.

Phycoerythrin and its chromophore groups were examined by Rudiger et al. (1967) using the chromic acid micro-degradation technique already described in the discussion of phycocyanobilin. They found that methylation of the free carboxyl groups of the "purple pigment" formed by refluxing phycoerythrin with methanol gave a product spectrally and chromatographically identical with aplysioviolin; also, aplysioviolin treated with 10N hydrochloric acid becomes spectrally identical with phycoerythrobilin, this latter name being applied to the pigment released from phycoerythrin on controlled acid hydrolysis. Furthermore, "purple pigment" phycoerythrin, phycoerythrobilin, and aplysioviolin all give a transient blue colour on treatment with alkali. The evidence thus suggests that "purple pigment" is the unesterified

form of aplysioviolins. The structure suggested for the "purple pigment" is shown in Fig. 2; its mono-methyl ester is the structure previously proposed for aplysioviolins.

Phycoerythrobilin rather than "purple pigment" appears to be the native prosthetic group of phycoerythrin. They have identical side-chains, and must be very closely related.

O'hEocha (1966) reported the isolation of two crystalline urobilins from phycoerythrobilin, one found by catalytic hydrogenation, the other by treatment with concentrated acid, and was able to show that these products were in fact derived from the phycoerythrobilin itself, and not from a phycourobilin pigment in the protein. Isolation of very small quantities of authentic phycourobilin, however, was claimed by Paul (1950) who hydrolysed chromopeptides resulting from tryptic digestion of R-phycoerythrin with silver sulphate to give a urobilinoid pigment free of amino-acids.

The Chromophore-apoprotein linkage.

The earliest suggestion as to the nature of the linkage between the chromophore group and the protein moiety came from Lemberg (1930). He postulated that since concentrated hydrochloric acid was at that time the only known reagent which would cleave the chromophore from the molecule, the linkage must be a peptide one, through the propionic acid side chains of the chromophore to amino-groups in the protein. O'Carra and O'hEocha (see O'hEocha, 1960) have shown that the ϵ -amino groups of lysine in R-phycoerythrin do not participate in such linkages; on the other hand, O'Reilly and Berns (1963)

have found that after treatment of C-phyococyanin from *Plectonema calothricoides* with fluorodinitrobenzene, there remained an unaltered lysine residue. All the tyrosine residues were found to be altered. O'Reilly and Berns (1963) suggested the possibility of a chromophore-protein peptide-type linkage, but a comparison of the rate of hydrolysis of the phycoerythrobilin linkage (O'Carra, 1962) with such comparable data as is available in the literature on the rates of hydrolysis of peptide bonds (see for example, Desnuelles and Casal, 1948) suggests that this particular prosthetic linkage is too labile to be a peptide-type bond. In his studies of the kinetics of the acid hydrolysis of phycoerythrin, O'Carra found that it was necessary to carry out the reaction in the presence of a large excess of ethyl mercaptan. If no mercaptan is present, the released chromophore tends to recombine with protein fragments to form artifact derivatives; the mercaptan competes with these protein fragments, and if present in sufficiently large amounts, reacts with virtually all the pigment to give a chloroform-soluble condensation product. O'Carra pointed out that none of the groups in the tetrapyrrolic ring system of phycoerythrobilin seems capable of participating in a stable acid-hydrolysable linkage; furthermore, participation of any of the oxygen- or nitrogen-containing groups of the tetrapyrrolic nucleus in the prosthetic nucleus would be expected to hinder zinc complex formation or to be reflected in a spectral difference between the zinc complexes of the free and bound pigments, which is not the case (O'Carra et al., 1964).

The only other functional groups of phycoerythrobilin are the carboxyl groups on the propionic acid side chains. These could form either ester or peptide-type linkages, although the latter are probably eliminated by the evidence discussed above. The relative acid lability of the prosthetic linkage is consistent with its being an ester-type linkage. The possibilities are a linkage or linkages from one or both propionic acid side-chains of phycoerythrobilin to a hydroxyl group or hydroxyl groups of the protein chain - for example, the hydroxyl groups of serine, threonine, or tyrosine. A further possibility is an ester linkage from an aspartic acid or glutamic acid carboxyl group to a hydroxyl group on the chromophore, formed by enolisation of a ring keto-group. The ease of cleavage of phycocyanobilin with potassium hydroxide solution would suggest an ester-type linkage (Chapman et al., 1968, in press). The publication of these last results is awaited with interest since Paterson (1967) found no evidence for the release of phycoerythrobilin from B-phycoerythrin at high pH values, although he reported the isolation of a number of chromopeptides from the reaction mixture.

Biosynthesis

It is only recently that any direct evidence has been obtained regarding the biosynthesis of biliproteins, and this is based mainly on studies of action spectra of their formation. It appears that the biosynthetic pathways of chlorophylls and the biliproteins are different. An ideal experimental organism for the study of phycocyanin biosynthesis is a heterotrophic mutant of the anomalous alga *Cyanidium caldarium*

which contains C-phycoerythrin and allophycoerythrin but no chlorophyll (Nichols and Bogorad, 1962). They concluded that a haem compound acts as photoreceptor and possibly also as precursor of C-phycoerythrin.

Fujita and Hattori (1960, 1962) worked with the Blue-green alga *Tolypothrix tenuis* which contains the biliproteins C-phycoerythrin, C-phycoerythrin and allophycoerythrin. They reported that bile pigment precursors of C-phycoerythrin and C-phycoerythrin are formed photochemically and that in the presence of nitrogen sources these are converted in the dark, through intermediates, into biliproteins. Bogorad (1963) has outlined two possible pathways which might operate in biliprotein biosynthesis, the second of which is better substantiated by the work of Fujita and Hattori. The biosynthesis of the bilichromoproteins of the Rhodophyta and Cyanophyta has been reviewed by Guerin-Dumartriat (1960). Troxler and Lester (1967) established that δ -aminolevulinic acid is a direct precursor of phycoerythrin, and also that porphobilinogen and coproporphyrinogen III are intermediates in the biosynthesis of phycoerythrin.

present work will now be discussed in greater detail.

(a) Paper chromatography. Three papers are involved in this form of chromatography: a moving liquid phase, a stationary phase at the surface of the cellulose sheet and a vapor phase which may or may not be in equilibrium with the liquid phase. The rate of migration of the moving liquid phase is a function of the first phase of the solvent properties of the solvent: namely, the viscosity, the surface tension and

EXPERIMENTAL SECTION

(a) General Techniques

Before going on to give the practical details of the various experiments carried out, it will be convenient to describe in a general manner some of the techniques used.

Chromatography. Chromatography has been defined as "the uniform percolation of a fluid through a column of more or less finely divided substance which selectively retards, by whatever means, certain components of the fluid," (Martin, 1950), and this forms a convenient general definition of the process provided the term "finely divided substance" is extended to include paper. The physico-chemical phenomena involved in the chromatographic process vary widely, and include adsorption, ion exchange, and partition between two phases, all of which are encountered in separations on columns or on paper. Often the distinction between the various types mentioned is quite arbitrary, as it cannot always be stated with certainty which of the above phenomena is operating in a given separation.

The various forms of chromatography employed in the present work will now be examined in greater detail.

(a) Paper chromatography. Three phases are involved in this form of chromatography: a moving liquid phase, a stationary phase at the surface of the cellulose fibres and a vapour phase which may or may not be in equilibrium with the liquid phase. The rate of migration of the moving liquid phase is a function in the first place of the physical properties of the solvent: notably the viscosity, the surface tension and

the density, all of which are dependent on the temperature. Secondly, the rate of migration depends on the pore size of the paper: very fine channels and capillaries will exert a greater suction force on the liquid than wider channels. Paper usually differs in structure along the two dimensions of its surface and solvent will usually rise faster up a strip cut in the machine direction.

The rates of migration of solutes are governed by specific partition between the moving liquid phase and the stationary gel-like phase which is usually considered to be located in the amorphous regions of the cellulose. Good paper is at least 96 per cent α -cellulose, and about 40 per cent is in the amorphous state. Most of the water is held by the amorphous regions; chemically bound water exists in native cellulose to the extent of about 5.9 per cent. Rates of migration of selected amino acids come close to the theory for simple liquid-liquid distribution, but the basic amino acids have a strong affinity for cellulose and specific adsorption or ion-exchange effects appear to operate. Migration rates are generally expressed in terms of the R_F value, which is the ratio of the distance travelled by the solute to the distance travelled by the solvent front. The R_F value is greatly affected by temperature variations; interactions between vapour and liquid phases caused by temperature variations during a run can be the cause of non-uniform operating conditions.

In this laboratory both one- and two-dimensional paper chromatography has been used. Two-dimensional amino acid chromatograms were run on Whatman No.1 paper, and the solvent

system used was (1) n-Butanol:Water:Acetic Acid 4:5:1, followed by (2) 90 per cent aqueous phenol (Solvent system A). Samples were spotted 12 x 12 cm. from the corner of a 45 x 45 cm. sheet, and run in the first direction with solvent (1) overnight. The chromatogram was then air-dried for 12 hours, retrimmed and run in the second direction in solvent (2). The chromatogram was then air-dried for a further 12 hours, washed twice in ether, and allowed to dry for a further 12 hours before development with ninhydrin. The ninhydrin was used as a 0.5 per cent solution either in water-saturated n-butanol (n-butanol containing approximately 11 per cent v/v water) or in pure ethanol. The latter solvent is preferable as the vapour of n-butanol is rather unpleasant. The chromatogram was dried in air and then heated in an oven at 80°C for 5-10 minutes to develop the colour; the colour could be intensified by exposing the chromatogram to a current of steam. The colours produced vary from pink to purple, except for proline which gives a yellow colour with ninhydrin. The reaction of α -amino acids with ninhydrin is a complex one, and has not yet been satisfactorily explained in all its details. The ninhydrin colour tended to fade after a few days, and could be stabilised by spraying with an ethanolic copper nitrate solution, prepared by diluting 1 ml. saturated cupric nitrate solution plus 0.2 ml. 10 per cent nitric acid (v/v) to 100 ml. with ethanol (Kawaran and Wieland, 1951). The spots then appear pink on a green background.

A typical amino acid map thus obtained is shown in

this layer plate. The first, manufactured by G. De Agostini, of

Diag. 3. In recent years considerable attention has been given to the problem of maximum resolution of amino acids by one-dimensional chromatography. The main drawbacks of two dimensional chromatography are:

- (1) only one sample can be separated for each run,
- (2) the amount of sample which can be used is limited,
- (3) the method is not ideal for quantitative work.

A large number of one-dimensional systems have been devised - these have been reviewed by Leggett Bailey (1962). That which found most use in the current work was ethyl methyl ketone:propionic acid:water 75:25:30 (Solvent B) (Clayton, 1954). The solvent was allowed to flow downwards for $7\frac{1}{2}$ hours, and the chromatogram dried and developed as above. R_f values for a number of amino-acids in this solvent are listed in Table 4. The Butanol:water:acetic acid solvent already mentioned was also used as a solvent in one-dimensional chromatography (Solvent C), and other solvents used will be described where relevant.

Thin layer chromatography. Thin layer chromatography is a technique which has evolved rapidly in the last twenty years. It has been of special importance for the analysis of lipids and proteins, and in this laboratory the substitution of cellulose thin layers for paper has been found to lead to greatly improved resolution of components. A variety of different materials have been used in thin layer chromatography; these include cellulose, cellulose acetate, starch and agar gel, silica gel, and acrylamide gel.

Two different sets of apparatus were used for spreading thin layer plates. The first, manufactured by C. Desaga, of

TABLE 4 ; R_f values of the common amino acids in solvent system B :- Ethylmethyl Ketone: Propionic Acid: Water 75:25:30.
(Clayton, 1954).

<u>AMINO ACID.</u>	<u>R_f VALUE.</u>
Leucine	0.81
Isoleucine	.78
Phenylalanine	.75
Tryptophane	.69
Methionine	.65
Valine	.62
Tyrosine	.57
Proline	.51
Alanine	.47
Glutamic Acid.	.44
Threonine	.43
Glycine	.39
Hydroxyproline	.39
Aspartic Acid.	.36
Serine	.34
Arginine	.30
Histidine	.28
Lysine.	.25
Cysteine	.15 (streaking).
Cystine.	.14

Heidelberg, was simple to use, and gave very satisfactory results, the main drawback being that the glass plates used must be of constant thickness, since any variations in the thickness tend to cause chipping of the edges of the plates as the spreading device runs over them, and also to non-uniformity in the thickness of the layer produced. These difficulties are overcome in the Shandon apparatus, which incorporates an air-filled pressure pad in its design to ensure that the upper surfaces of all the plates are on the same level. In fact use of the Shandon apparatus was found to be time-consuming, and considerable care was required in setting up the apparatus if satisfactory results were to be obtained, and consequently the Desaga apparatus was generally used in spite of the disadvantages mentioned. Two sizes of plate were used: 20 x 20 cm., or 5 x 20 cm.; the apparatus was such that five 20 x 20 cm. plates, or an equivalent number of 5 x 20 cm. plates, could be spread at once. Silica gel and cellulose were the materials used for spreading the plates: the slurries of these materials used were prepared as follows, in each case the quantities being sufficient for five 20 x 20 cm. plates of layer thickness 250μ :

(a) Silica gel: MN Kieselgel G-HR (Macherey, Nagel and Co. Ltd.) (30gm.) was shaken with distilled water (70ml.) for 90 seconds.

(b) Cellulose powder: MN cellulose powder 300 (Macherey, Nagel and Co. Ltd.) (15gm.) was mixed with distilled water (70ml.) and ethanol (10ml.), and "sonicated" for 1 minute to ensure thorough mixing: "Sonication" - agitation by a source of ultrasonic sound - will be described at a later stage in

this section. It is important that the slurries thus prepared are used immediately. The plates were cleaned with chromic acid before use. Cellulose plates were found to be more useful for the samples used; a variety of solvents were used and these will be mentioned in the text as appropriate, but it will be convenient to mention here two two-dimensional systems which were used for the resolution of amino acid mixtures:

- (1) Propan-2-ol:Formic acid:water 40:2:10(v/v)
- (2) t-Butanol:ethyl methyl ketone:0.88 ammonia:water
5:3:1:1(v/v) (Solvent system D).

The tank atmospheres were unsaturated with respect to solvent vapour before development of the chromatogram; it was found (Von Arx and Neher, 1963) that prior saturation of the tank atmosphere led to inferior separation of the amino acids. Each solvent was run for about 4 hours (about 13 cm. movement of the solvent front), the plate being dried and the yellow "impurity" band at the solvent front being removed between the runs. The plate was finally dried in warm air prior to development with ninhydrin. The saving in time compared with the paper chromatography system already described is immediately apparent; there is also an improvement in resolution in spite of the smaller overall size of the chromatogram. A typical map obtained with this system is shown in Diagram 4.

The second solvent system used was designed specifically for the detection of the amino acid homoserine (2-amino-4-hydroxy-butyrac acid) in the presence of other neutral amino acids. The two solvents which were found to give satisfactory

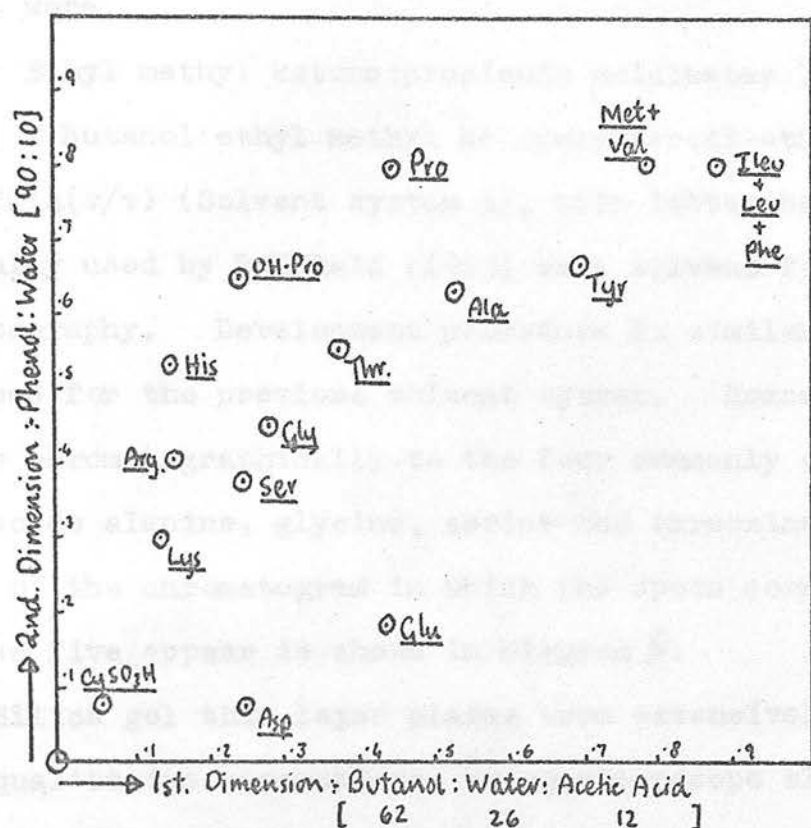


DIAGRAM 3: Two-dimensional chromatography of amino acids using solvent system A on Whatman No.1. Paper.

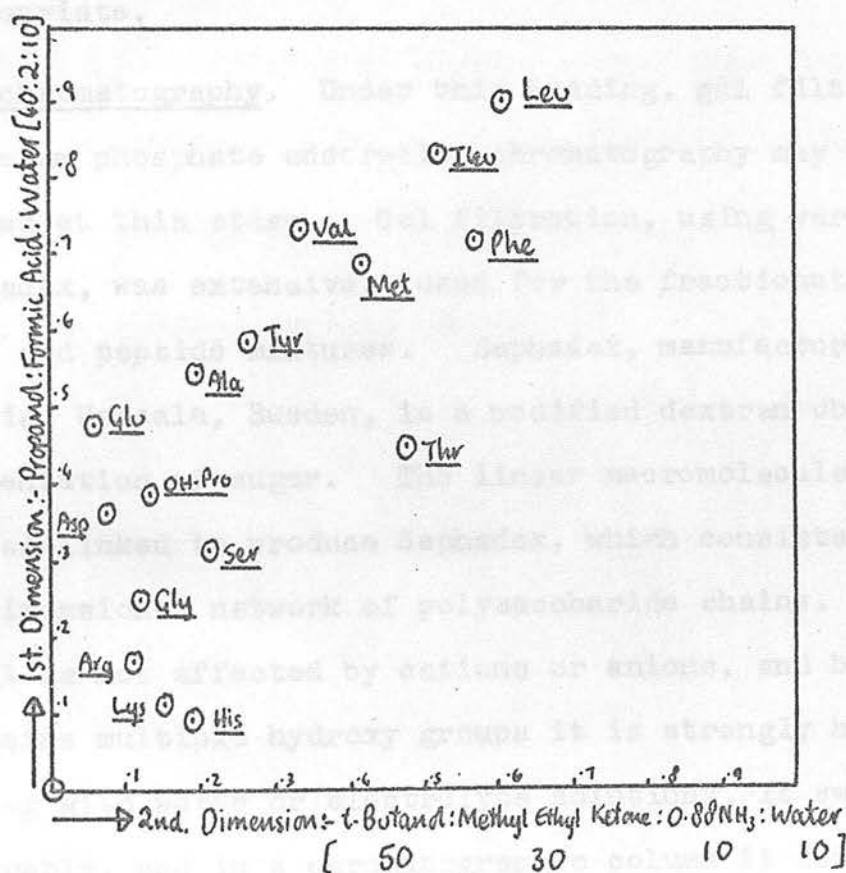


DIAGRAM 4: Two dimensional chromatography of amino acids on cellulose thin layer plates, using solvent system D.

results were

(1) Ethyl methyl ketone:propionic acid:water 75:25:30(v/v) and (2) t-Butanol:ethyl methyl ketone:water:di-ethylamine 40:40:20:4(v/v) (Solvent system E), this latter being originally used by Redfield (1953) as a solvent for paper chromatography. Development procedure is similar to that described for the previous solvent system. Homoserine is similar chromatographically to the four commonly occurring amino acids alanine, glycine, serine and threonine, and the region of the chromatogram in which the spots corresponding to these five appear is shown in Diagram 5.

Silica gel thin layer plates were extensively used for rapid qualitative separations, using microscope slides dipped in a suspension of silica gel in chloroform. A variety of solvents were used and these will be described in the text as appropriate.

Column chromatography. Under this heading, gel filtration and calcium phosphate adsorption chromatography may be discussed at this stage. Gel filtration, using various forms of Sephadex, was extensively used for the fractionation of protein and peptide mixtures. Sephadex, manufactured by Pharmacia, Uppsala, Sweden, is a modified dextran obtained by fermentation of sugar. The linear macromolecules of dextran are cross-linked to produce Sephadex, which consists of a three-dimensional network of polysaccharide chains. The material is not affected by cations or anions, and because it contains multiple hydroxy groups it is strongly hydrophilic. On mixing with water or electrolyte solutions, it swells considerably, and in a chromatographic column it acts as a

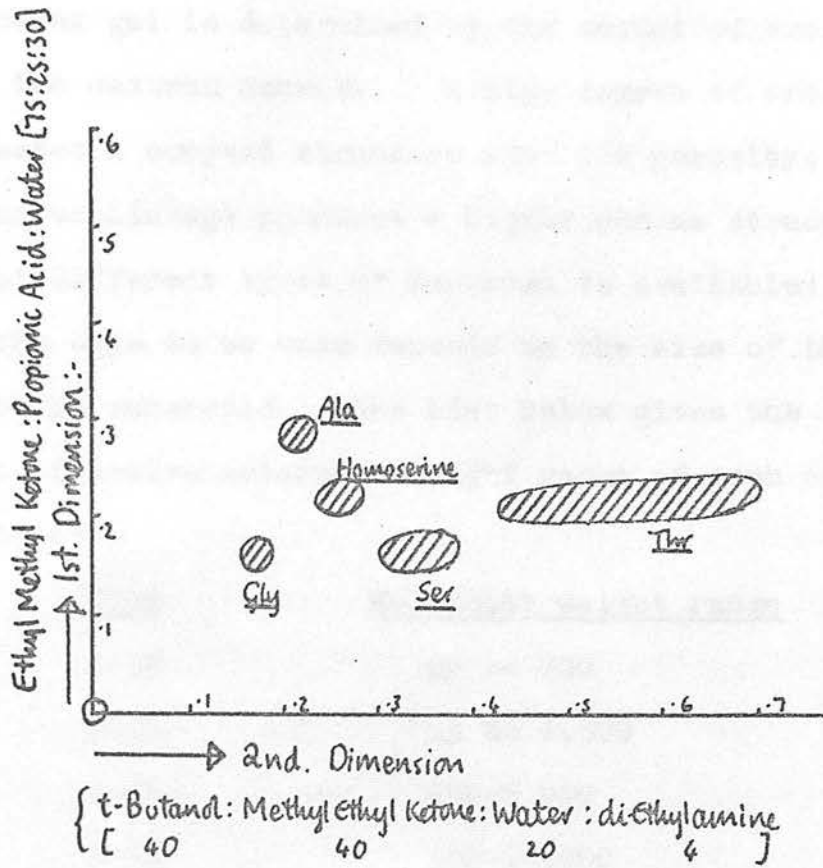


DIAGRAM 5: Two dimensional Cellulose thin layer chromatographic system devised to identify homoserine in the presence of other neutral amino acids (only a part of the chromatogram is shown).

sieve for molecules of different sizes, since the porosity of the Sephadex gel is determined by the amount of cross linkage in the dextran network. A high degree of cross linkage creates a compact structure with low porosity; a low degree of cross linkage produces a highly porous structure. A variety of different types of Sephadex is available; the choice of the type to be used depends on the size of the molecules to be separated. The list below gives the approximate effective molecular weight range of each of the available types:-

<u>Type</u>	<u>Molecular weight range</u>
G-10	up to 700
G-15	up to 1,500
G-25	100-5,000
G-50	500-10,000
G-75	1,000-50,000
G-100	>1,000-100,000
G-200	>1,000-200,000

These figures can only be approximate, since fractionation depends not only on the size of the molecules to be separated, but also on their shape and chemical structure.

Larger molecules that cannot enter the gel pores move through the column faster and thus become separated from the smaller ones. Once the smaller molecules have been washed through, the column is ready for a further separation; no regeneration is necessary.

Columns were packed in accordance with the manufacturer's instructions ("Sephadex" literature); the buffer systems used will be described in the text.

The use of calcium phosphate columns for the purification of proteins has been discussed at length by Swingle and Tiselius (1951) and Tiselius (1954). Although precipitated calcium phosphate is crystalline in structure, as shown by X-ray patterns, it is nevertheless a gel of high water content. The most stable form is hydroxyl-apatite, $\text{Ca}_5(\text{PO}_4)_3\cdot\text{OH}$, which was prepared by the method of Siegelman et al. (1965a), in which 0.5M solutions of calcium chloride and disodium hydrogen phosphate were allowed to run slowly into a beaker simultaneously with stirring; the precipitated brushite was washed four times with distilled water by decantation, and then boiled with an equimolar quantity of 0.25N sodium hydroxide solution for one hour. The precipitate was washed with distilled water a further four times, and then with 0.01M sodium phosphate buffer, pH 6.5, until the pH of the buffer remained constant. The hydroxyl apatite was stored in 0.001M sodium phosphate buffer of pH 6.5. Columns were prepared by pouring a suspension of the adsorbent in buffer into glass columns of suitable size.

During adsorption chromatography, tailing tends to occur unless the composition of the eluting solution is such that the R_f of a given solute approaches unity. Successful fractionation of a protein mixture depends on the use of buffer concentrations sufficiently far apart in ionic strength to provide clean cut elution of the components of the mixture from the column. The use of such columns for the separation of the algal biliproteins has been described by Haxo et al. (1955).

Electrophoresis. Under this heading may be considered:

(a) Paper electrophoresis and high voltage electrophoresis.

(b) Thin layer electrophoresis.

(c) "Disc" electrophoresis.

(a) Paper electrophoresis is a very useful and rapid technique for the preliminary examination of peptide mixtures; for preparative work and for fine analytical separations of macromolecules it has been almost entirely superceded by other inert materials. The main physical factors affecting migration rates are (i) the type and concentration of the buffer (ii) electro-endosmosis (solvent flow induced by the electric field and (iii) the electric field itself.

(i) The nature of the components of the buffer solution will influence the size and shape of the moving zones. The various effects which may be encountered have been discussed by Edward (1958). The pH of the background buffer will determine the net charge of the migrating ion; a particle with a net positive charge migrates towards the cathode, while one with a net negative charge migrates towards the anode. For a protein, peptide or amino acid, electrophoresis carried out at a pH which coincided with the iso-electric point would result in no net movement of the sample. Electrophoresis at a lower pH would lead to movement towards the cathode, and at a higher pH, movement towards the anode would result.

(ii) During paper electrophoresis the immobile paper phase, being negatively charged, causes what is known as an "endosmotic flow" of solution towards the cathode. Although it is possible to change the polarity of the paper by chemical means (Jermyn and Thomas, 1953), the amount of the flow is not intolerable under normal conditions, and no

correction was made for it in the present work.

(iii) The voltage used in paper electrophoresis represents a compromise between two conflicting features - it must be high enough to bring about reasonably large movements of sample, and at the same time not so large that reproducible results are not obtainable due to such factors as overheating, associated with high voltages and currents.

The apparatus used in the present work was constructed of Perspex and was basically the same as that of Grassman et al. (1951). The paper strip (26cm. x 15cm.) was spotted with sample across the centre line, and placed with its ends dipping in the anode and cathode buffer compartments, being supported between the buffer compartments by a rigid polythene sheet. The grades of paper used included Whatman Nos. 4, 54 and 3MM, with a voltage gradient usually around 20-25v. per cm. The buffers used are listed in Table 5; electrophoretograms were normally examined under ultra-violet light prior to development with ninhydrin.

Although useful separations can be obtained using the order of potential gradient mentioned above - around 25v. per cm. - the high diffusion rates of low molecular weight compounds render unsatisfactory attempts to separate complex amino-acid or peptide mixtures under these conditions. However, this problem may be overcome by the use of high voltage electrophoresis, in which potential gradients of 50-200v. per cm. are used. The main problems encountered in the use of such voltages are connected with the safety both of the sample and of the operator.

The apparatus used was a Miles Hivolt Electrophoresis

Unit Mark III. This instrument is designed to operate at up to 10kV., delivering a current of up to 500mA., and it incorporates a number of trip circuits designed to ensure the safety of the operator and to prevent damage to the instrument. These circuits guard against such eventualities as short circuits to earth, overloading, cooling water supply failure, or accidental opening of the lid during a run.

The paper used in the apparatus was Whatman Nos. 1, 54, or 3MM; the strip length was approximately 45cm., and the width of the strip was chosen according to the sample size. The buffers used are listed in Table 5. The electrophoretograms were developed as before.

(b) Thin layer Electrophoresis. The principle of this technique is the same as that outlined for paper electrophoresis, with thin layer plates replacing paper strips as the immobile phase. The method of spreading the plates has already been described; silica gel and cellulose plates were used, and an aerosol-powered buffer spray was used to moisten the plates to avoid damage to the plate surface. The Desaga Thin-layer Electrophoresis unit, which incorporates a cooling surface, was used; similar development procedures to those already described were used. The main advantage of thin layer plates over paper is the better resolution obtained; the disadvantage is the time involved in preparing the plates.

(c) Disc Electrophoresis. This is the name popularly applied to zone electrophoresis in gels, and is derived from the discontinuous buffer systems used. Gel electrophoresis differs from simple zone methods (for example, electrophoresis on paper or thin-layer) in that the medium is not inert, but

TABLE 5. Solvents and Buffers used in Chromatography and Electrophoresis.

(a) Chromatography.

No.	Components	Proportions.	Reference.
A	n-Butanol : Water : Acetic Acid. Phenol : Water	62:26:12 90:10	
B	Ethyl Methyl Ketone : Propionic Acid : Water	75:25:30	Clayton, 1954
C	n-Butanol : Water : Acetic Acid	62:26:12.	
D	Propan-2-ol : Formic Acid : Water t-Butanol : Ethyl Methyl Ketone : 88 Ammonia : Water	40:2:10 5:3:1:1	Von Arx and Neher, 1963.
E	Ethyl Methyl Ketone : Propionic Acid : Water t-Butanol : Ethyl Methyl Ketone : Water : di-Ethylamine	75:25:30 40:40:20:4	Clayton, 1954 Redfield, 1953

(b.) Electrophoresis.

No.	Components.	Proportions.	pH.
F	Water : Pyridine : Acetic Acid	290 : 8 : 2	5.6
G	Water : Pyridine : Acetic Acid	290 : 6 : 4	5.1
H	Water : Pyridine : Acetic Acid.	290 : 4 : 6	4.5
I	Water : Pyridine : Acetic Acid	289 : 1 : 10	3.6
J	Acetic Acid : Water	5 : 95	2.4
K	Acetic Acid : Formic Acid : Water	4 : 1 : 95	2.2.



is deliberately designed to interact with the migrating proteins to produce a sieving effect. The gel can be "tailored" by varying the gel strength to suit the size of the molecules being separated. Smithies (1955, 1959), one of the pioneers of gel electrophoresis, used strong starch gels, but more recently polyacrylamide gels have come into favour (Raymond and Weintraub, 1959; Raymond, 1964; Ornstein and Davies, 1964). These have the advantages of being quick to prepare and easy to use; also they may be prepared in a much wider range of pore sizes than starch gels, and they may be used in a much wider range of buffers.

The apparatus used was the Shandon Basic Outfit for Disc Electrophoresis. In this, vertical columns of polyacrylamide gels are formed in situ in precision bore glass tubing. These columns consist of a small pore gel layer occupying most of the tube, and a large pore layer which is formed above it. The sample, applied to the top of the large pore layer, migrates rapidly through the large pore gel under the influence of the electric field, but is slowed by the restrictive properties of the small pore gel, and is concentrated to a narrow starting zone at the interface between the two gels. Separation then proceeds in the small pore gel as a combination of electrophoresis and gel filtration. After electrophoresis the gel column is removed from the tube and stained with one of a number of ionic dyes - naphthalene black was used in this laboratory - and excess dye washed away. The components of the mixture may then be observed.

The buffer-gel system used had a pH of 6.7, the gels and

buffer being prepared in accordance with the instructions in the Shandon instruction manual; difficulty was experienced in bringing about satisfactory polymerisation of the large pore gel, however, and accordingly samples were loaded on to the top of the small pore gel column by the method of Broome (1963), in which an aqueous solution of the protein or peptide sample is mixed with a suspension of Sephadex G-200 in a "tris"-HCl buffered sucrose solution, and the mixture is carefully added to the top of the column. A current of approximately 5mA per tube was passed, generally for 60-90 minutes, a voltage of about 200v. being required.

Evaporations. Evaporations were normally carried out using a rotary evaporator. Two such evaporators were used:

(a) a Buchi "Rotavapor" rotary vacuum evaporator, used in conjunction with a water-bath and a water-pump. Evaporations of solutions other than amino-acid hydrolysates were carried out at less than 40°C unless specifically stated otherwise.

(b) Vacuum Evaporator, Jones and Stevens Type VW, which has the advantage that it can be used in conjunction with an oil-pump for the removal of such solvents as amyl alcohol whose boiling points are such that they are not easily removed with a water-bath.

Dialysis. Dialysis is basically a molecular sieve technique, used mainly for the removal of small molecules from aqueous solutions of proteins and other high molecular weight solutes and for the concentration of protein solutions. In this laboratory the usual procedure was to wash dialysis tubing (Cellulose or Visking) thoroughly with distilled water, and

then to tie both ends closed with the sample inside, room being left inside the tube to allow for expansion due to osmosis. Dialysis was either carried out against running tap water or against repeated changes of distilled water or buffer solution. In the two latter methods, a small quantity of toluene was added on top of the distilled water or buffer to inhibit microbial action. Dialyses were carried out, whenever possible, in the cold room (5°C). It should be noted that dialysis was not used to desalt protein samples on which carbohydrate estimations were to be carried out since glycerol, present in small quantities in dialysis tubing, tends to contaminate the protein and give false carbohydrate readings.

Freeze-drying. Freeze-drying is a very efficient method for concentrating protein or peptide solutions. The solution is frozen and exposed to a high vacuum in the presence of a substance which binds water.

Sublimation of the ice from the surface of the frozen solution occurs, leaving the protein or peptide as an air-dry powder. Most proteins are thus obtained in the native state, although some proteins are irreversibly denatured by freezing.

Two freeze-driers were used in the course of the present work: these were Centrifugal Freeze-drier Model 30 Pl/599, and Freeze-drier Model 10P, Serial No. 440, both manufactured by Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex. Samples were frozen by placing in round-bottomed flasks, immersing these in liquid nitrogen, and rotating them so that the frozen solution was spread round the walls of the flasks,

thus presenting a large surface area. Freeze-drying was generally found to be complete within a period of twelve hours.

Ultrasonication. Ultrasonication is the name used for the treatment of a sample with a source of ultrasonic vibrations. This was used primarily for the rupture of the cell walls of the algal material to release their protein content into solution. It was also used for the agitation and mixing of the slurries used for spreading thin-layer chromatography plates, a point already mentioned. The instrument used was a Dawes Soniprobe (Type 1130A), manufactured by Dawe Instruments Ltd., London, England. Samples thus treated were found to heat up due to the amount of energy available, and the vessels containing them were therefore surrounded by an ice-bath. This is especially important when dealing with proteins which are very easily denatured irreversibly by heat.

Spectrophotometry. Ultra-violet and visible spectra were recorded on a Perkin-Elmer 137 UV automatic spectrophotometer, or on a Unicam SP800. Where absorptions at only one or two wavelengths were required a Unicam SP500 or 600 was used.

Infra-red spectra were recorded on a Unicam SP200 instrument.

Nuclear-magnetic resonance spectra were recorded on a Perkin-Elmer R10 60 m/cs. instrument.

Amino Acid Analysis. The search for a reliable, speedy and simple technique for the analysis of amino acid mixtures has ranged from straightforward colorimetric analysis, limited to a range of only a few amino acids, through paper chromato-

graphy which, although wider in scope, lacks resolution and reproducibility and requires extreme delicacy of technique on the part of the operator. Gas phase chromatography has the disadvantages of lack of accuracy, multiplicity of columns, and inapplicability to samples containing proteins or polypeptides. Micro-biological methods are tedious and unwieldy.

Column chromatography using columns of 8 per cent cross-linked polystyrene sulphonic acid resin provides accurate fractionation by cationic exchange. The affinity of each acid for the resin in the presence of a buffer of known pH and ionic strength results in a characteristic delay in its migration down the column, achieving a degree of separation dependent solely on column length. The necessity for the collection of large numbers of fractions for colorimetric analysis is avoided by automatically mixing the eluate from the column with a ninhydrin solution; the colour is developed by passing the mixture through a heated coil, and measured by photoelectric colorimeters which record the results on a moving chart.

The apparatus used in the present work was the Technicon Automatic Amino Acid Analyser. The method used was that of Spackman et al. (1958) as modified by Benson and Patterson (1965). The columns used were normally of Amberlite CG120 (type III), fractionated by the method of Hamilton (1958). Long columns (60 x 0.636cm.) were used for the fractionation of acidic and neutral amino acids; short columns (16 x 0.45 cm.) were used for the fractionation of basic amino acids. For the analysis of hydrolysates derived from reduced peptides where it was necessary to estimate homoserine, a long column

(58 x 0.636cm.) of Permutit Zeo-Karb 225 x 12, bead diameter 12.5μ , was used.

Hydrolysis of samples for analysis was carried out using 6N (constant boiling) hydrochloric acid (6ml.) in a sealed Pyrex tube, the tube being cooled in liquid nitrogen and evacuated at the water-pump prior to sealing. Hydrolysis was carried out in an oven at 105°C for 24 hours; after this time the tube was again cooled in liquid nitrogen and opened. The contents were transferred to a pear-shaped flask and evaporated to dryness on the rotary evaporator at 70°C ; water was added and the evaporation repeated, and this last process was carried out twice more. The residue was then dried overnight in vacuo over sodium hydroxide pellets. It was then dissolved in the appropriate amount of pH 2.2 buffer (0.0667M. w.r.t. sodium citrate) containing as internal standards norleucine (NOL) and L- α -amino- β -guanido-propionic acid hydrochloride (AGPA), both at a concentration of $0.1\mu\text{M}$ per 0.5ml. buffer.

Desalting of peptide samples which had been reduced with lithium borohydride and contained large amounts of salts, particularly lithium chloride, was carried out on an electro-dialysis apparatus of the type described by MacPherson (1946). The apparatus is made of Perspex and has three compartments; the central compartment contains the sample in aqueous solution and the anode and cathode compartments, separated from the central compartment by formolized gelatin on a cloth base and vegetable parchment respectively, each contain distilled water; the electrodes are made of platinum foil. The nature of the apparatus is such that only large peptides

carrying little or no net electric charge at the pH used may be desalted; smaller, highly charged peptides and free acidic or basic amino acids tend to migrate to the outer compartments. Passage of a current leads to migration of the various ions present. The progress of the desalting may be followed by an ammeter inserted in the circuit; when the process is complete, as indicated by a very low or zero reading on the meter, the contents of the central compartment are evaporated to dryness, giving the salt-free peptide.

those which develop thick walls and become heterocysts or spores, which are formed in most species of the family (*Sp. 1957*)

Anabaena differs from *Nostoc* only in that *Anabaena* forms no firm colonies. Plants of *Nostoc* - many of which attain relatively large size - are spherical or cushion-shaped gelatinous masses in which the trichomes are scattered or intertwined. Plants of *Anabaena* are aquatic, microscopic and visible to the naked eye only where they grow on algae. The trichomes are straight or spiralled, rigid and fragile; the spores are large and the sheath, which is interrupted in the case of *Anabaena cylindrica*, is composed of a pectic material, and is readily hydrolysed and often completely dispersed. *Nostoc* is a true soil-growing species; its distribution is world-wide, and it grows especially well on wet tropical soils. *Anabaena* also is found on soil surfaces, although both it and *Nostoc* grow in water. Both species are nitrogen-fixing, that is, they can utilise atmospheric nitrogen: this phenomenon is found only in genera forming heterocysts. (*Sp. 1957*; *Levin, 1962*).

CULTURE OF ALGAE.

Two different algae were examined as possible sources of C-phyococyanin. These were *Nostoc muscorum* and *Anabaena cylindrica*, which are both members of the Nostocaceae, which in turn are Cyanophyceae, or Blue-green algae. The Cyanophyceae chiefly form macroscopic layers or cushions on soil, rocks, wood and other objects; some form gelatinous masses of various shapes floating in water. Plants of the species Nostocaceae have barrel-shaped or depressed-spherical shaped cells, which divide in unison during periods of growth, except those which develop thick walls and become heterocysts or spores, which are formed in most species of the family (Smith, 1951).

Anabaena differs from *Nostoc* only in that *Anabaena* forms no firm colonies. Plants of *Nostoc* - many of which attain relatively large size - are spherical or cushion-shaped gelatinous masses in which the trichomes are contorted or intertwined. Plants of *Anabaena* are aquatic, microscopic and evident to the naked eye chiefly where they grow en masse. The trichomes are straight or spiralled, rigid and fragile; the spores are large and the sheath, which is interrupted in the case of *Anabaena cylindrica*, is composed of a pectic material, and is readily hydrolysed and often completely dispersed. *Nostoc* is a true soil-growing species; its distribution is world-wide, and it grows especially well on wet tropical soils. *Anabaena* also is found on soil surfaces, although both it and *Nostoc* grow in water. Both species are nitrogen-fixing, that is, they can utilise atmospheric nitrogen; this phenomenon is found only in genera forming heterocysts. (see Smith, 1951; Lewin, 1962).

The axenic growth of the two algae was carried out in suspension in the culture medium of Allen and Amon (1955), who originally used it for the growth of *Nostoc muscorum*, *Nostoc variabilis*, and *Anabaena variabilis*, all of which are nitrogen fixers; no combined nitrogen was added. The composition of this medium is as follows:-

MgSO_4	246mg.
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	110mg.
NaCl	234mg.
K_2HPO_4	348mg., all amounts being per litre of medium.

When the medium was sterilised by autoclaving, it was necessary to dissolve and autoclave the K_2HPO_4 solution separately to avoid precipitation of calcium and magnesium phosphates.

The following trace metal solution was added to the above medium at the rate of 5ml. per litre; when the medium was being autoclaved, the trace metal solution was added to that portion which did not contain the phosphate.

$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	200mg.	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	20mg.
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	125mg.	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	8mg.
H_3BO_3	285mg.	$\text{VSO}_4 \cdot \text{H}_2\text{O}$	3.5mg.
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	5mg.	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	4mg.
$\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$	1.8mg.		

2mg. iron was added in the form of its EDTA complex:

240mg. $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ were dissolved in 0.05M Na_2EDTA , and the solution, its pH adjusted to 7.6, made up to 100ml. 3.33ml. of this solution then contained 2mg. of iron.

The trace metal solution was then made up to 500ml.

with distilled water and added to the culture medium as required. The solution was kept in a dark glass bottle when not required, as exposure to light led - over a period of weeks - to a colour change from pale yellow to violet, presumably due to the formation of a permanganate by oxidation of the manganese sulphate.

Stock cultures of the two algae were maintained on agar slides, prepared in Pyrex screw-capped test-tubes using the culture medium described above containing in addition 1.5% Difco Bacto-Agar.

Culture of the algae was carried out in 250ml., 500ml., 1 litre, and 2 litre Pyrex conical flasks. 250ml. and 1 litre conical culture flasks with plastic screw-caps were available, although these had the inherent disadvantage that the caps prevented the circulation of air and carbon dioxide, over the medium. The non-absorbent cotton-wool stoppers used in the other flasks were found to give more satisfactory results. For large-scale cultures, which were carried out in 20-litre aspirators with approximately 14 litres of medium, air containing 5 per cent carbon dioxide was bubbled through the medium under sterile conditions at the rate of 1 litre per aspirator per minute. The tube leading the air into the culture vessel and the outlet tube were passed through a sterile rubber stopper in the neck of the aspirator, to prevent mould spores etc., from the atmosphere entering the flask.

Two methods of sterilising the culture medium were used - (a) autoclaving and (b) sterile filtration.

(a) For autoclaving, the cotton-wool stoppers were protected by a brown paper hood tied on with string, and the rubber stoppers used in the large culture vessels were wrapped in brown paper before being placed loosely in the necks of the aspirators. This prevented the stoppers from sticking. Culture vessels and medium were autoclaved for twenty minutes at 15lbs. per sq. in. above atmospheric pressure - 121°C - conditions normally regarded as sufficient to kill any organisms present. As was mentioned above, it was necessary to autoclave the medium in two portions, which had to be mixed after sterilisation, with the risk of contamination of the medium during the process of mixing, and moulds were frequently found growing in the media during culture. As an attempt to solve this problem, sodium glycerophosphate was used instead of di-potassium hydrogen phosphate, as glycerophosphates tend to be more soluble than the corresponding phosphates. This experiment, however, was not successful, as the new medium proved to be barely capable of sustaining the growth of the algae. An alternative method of sterilising the medium was therefore sought, and sterile filtration was found to be the solution to the problem.

(b) Sterile filtration. This process was carried out using the Millipore apparatus, manufactured by the Millipore Filter Corporation, Bedford, Massachusetts. Millipore filters are cellulose acetate porous membranes, produced with several different distinct pore sizes. A given membrane retains on its surface, from all liquids (or gases) passed through it, all particles, bacteria, and cells which exceed in dimension the filter pore size. The procedure

adopted was to sterilise the vessels and filter unit, containing a filter membrane of pore size 0.22μ , by autoclaving in the usual manner. The culture medium was then prepared, and pumped through the filter unit using a peristaltic pump. Care was taken at all times to avoid possible contamination, especially of the Pyrex ball-joints used to connect the filter unit to the culture vessels. These were heated briefly in a Bunsen flame immediately before use, to destroy any organisms present.

Anabaena cylindrica and *Nostoc muscorum* were obtained in bacteria-free cultures, nos. 1403/2 and 1453/8 respectively, from the "Culture Collection of Algae and Protozoa," the Botany School, Downing Street, Cambridge, England. These cultures were used to inoculate agar slides, which were in turn used to inoculate 250ml. conical flasks, 1 litre conical flasks, and finally 20 litre aspirators. Wire loops were used to transfer material to and from agar slides; the loops were flamed before use to sterilise them, as were the mouths of the tubes and flasks.

The inoculated cultures were then placed on a white surface and continuously illuminated from above with two Philips "Cool White" fluorescent lamps (M.C.F.E.; 80W/33). The culture room was maintained at a temperature of 22°C by means of a thermostatically controlled heater. The conical flasks were swirled daily to promote the circulation of air; the contents of the large vessels were agitated by the current of air and carbon dioxide passing through them.

The culture of *Nostoc muscorum* was discontinued after a short time due to its extremely slow rate of growth, which

was presumably caused by a deficiency in the culture medium or by unsuitable lighting. The observation that *Anabaena cylindrica* favoured growth on the walls of the vessel led to glass wool being placed in the larger vessels, thus providing a greater surface area for the growth of the alga, and this was found to result in an increased rate of growth.

After an average of four weeks' growth time, the alga from the large vessels was harvested. This was considered to be the most suitable time for harvesting, since cell lysis resulting in leaching of the biliproteins into the surrounding medium tended to occur if the cultures were left much longer.

Harvesting of the Alga, and extraction of the Biliproteins.

The harvesting procedure was as follows: The major part of the culture medium was withdrawn by suction through the sintered glass filter on the end of the air inlet tube, and discarded. The residual material, consisting of the glass wool plus the alga, was removed from the culture vessel, and the strands of glass wool reduced in length with a pair of scissors, which made for easier handling of the material, which was then suspended in water and subjected to sonication, as already described, for a period of fifteen minutes. The suspension was then centrifuged at 1000g. for twenty minutes. The solid material was suspended in water again, re-sonicated, and re-centrifuged. The two aqueous extracts were combined, and the solid material containing the glass wool and insoluble cell fragments was discarded. To the aqueous extract which contained the required biliproteins as well as chlorophyll and other soluble constituents of the algal cells, 40 per cent w/v ammonium sulphate was added slowly with stirring,

and the material thus precipitated was separated by centrifugation at 1000g. for twenty minutes, the clear solution of ammonium sulphate being discarded. The solid material was stirred with water and the cloudy, greenish-blue solution thus obtained was filtered through a short column of unsedimented celite, to remove chlorophyll, which formed a green band at the top of the column. The filtrate was blue with deep red fluorescence due to the presence of the required C-phycoerythrin and allophycoerythrin. A UV./vis spectrum of the protein solution at this stage is shown in Fig.5. The solution was then dialysed against running tap-water overnight to remove any residual ammonium sulphate, and then for four hours against 0.01M phosphate buffer, pH 6.1. One per cent (w/v) sodium chloride was then added; this has been found (Haxo et al., 1955) to promote adsorption of the proteins on the calcium phosphate column. The protein solution was loaded on to a 5 x 20cm. basic calcium phosphate column which had been equilibrated with 0.001 molar sodium phosphate buffer of pH 6.1, and eluted with a series of phosphate buffers of pH 6.0, and of increasing molarity (0.001M, 0.005M, 0.01M, 0.02M, 0.05M), about 250 mls. of each being used. No phycoerythrins were found in the protein mixture - these would normally be eluted from the column before the phycoerythrins. The C-phycoerythrin was eluted from the column by 0.05M phosphate buffer, giving approximately 150mls. of a blue solution with a pronounced dark red fluorescence. A small quantity of dark blue allophycoerythrin remained adsorbed at the top of the column; this was eluted by 0.2M sodium phosphate buffer.

The ultra-violet and visible absorption spectra of the

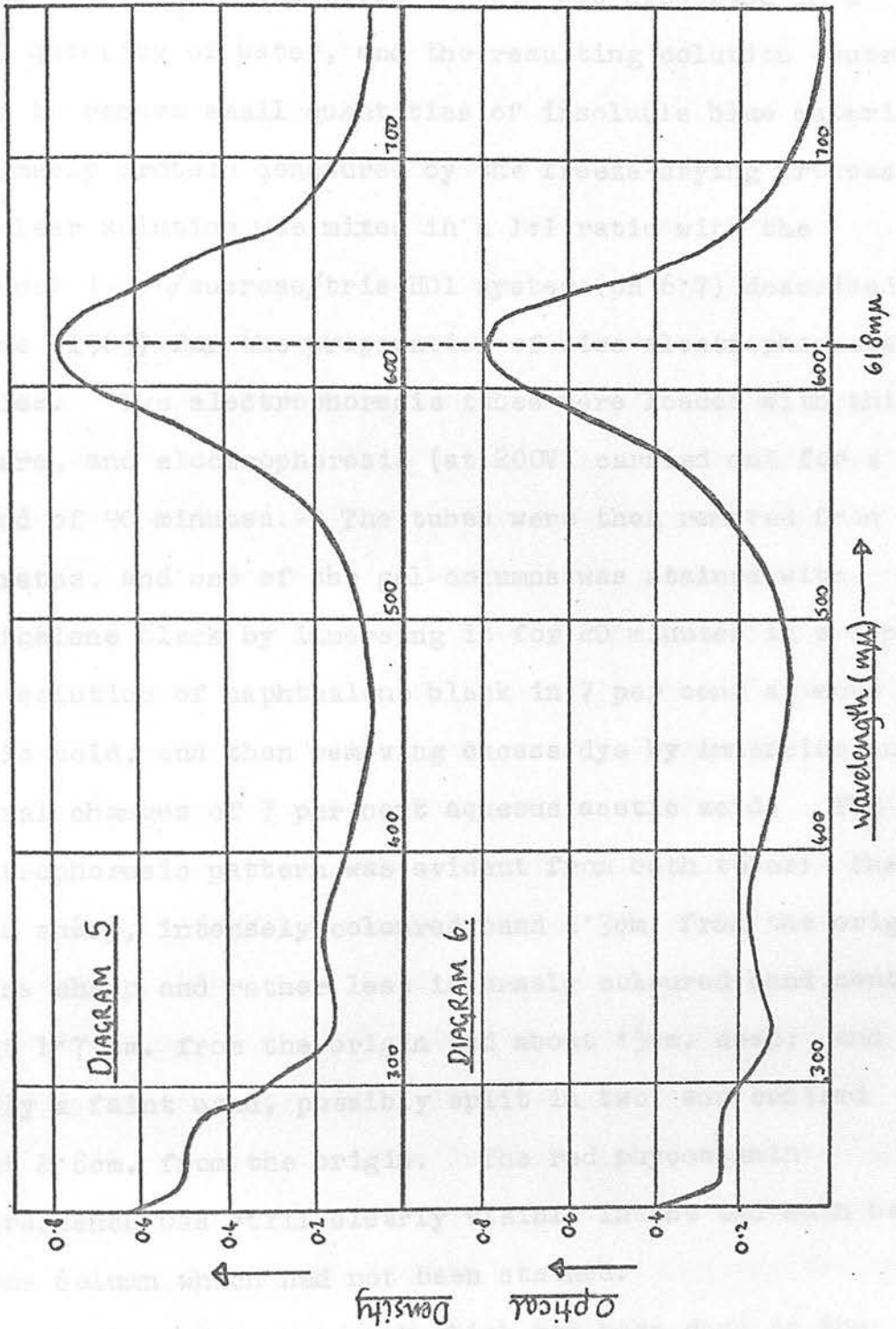
purified C-phycoerythrin solution are shown in Fig.6. The "spectral ratio" (ratio of the absorbance at 618m μ to that at 278m μ), which serves as a rough criterion of the purity of the protein, of the protein thus purified, was generally found to be in the region 3.3 - 4.0.

A typical harvest by the method outlined above yielded, from two 20 litre culture vessels, around 100mg. purified C-phycoerythrin. This was either stored under 40 per cent ammonium sulphate solution, or the solution was dialysed to remove the phosphate buffer, and freeze-dried. Freeze-dried material was stored in a desiccator until required.

Purity of C-phycoerythrin samples.

A sample of C-phycoerythrin which had been stored under 40 per cent ammonium sulphate solution was isolated by centrifugation, dissolved in a small amount of distilled water, and the solution dialysed overnight against running tap water to remove any remaining ammonium sulphate. The solution was then dialysed against 0.01M sodium phosphate buffer of pH 6.5 for four hours, and was then loaded on to a 3.2 x 45cm. Sephadex G-100 column, and eluted upwards with the same phosphate buffer. The protein sample was found by this method to be virtually homogeneous - only a small amount of "tailing" after the eluted coloured band indicated any lack of homogeneity. The ultra-violet and visible spectra of the protein sample (see Fig.6) used for the experiment and that of the purified material were virtually identical, indicating that the protein is virtually homogeneous. The "spectral ratio" of the purified protein is rather low - about 3.3 - and it is probable that some deterioration of

Absorption spectra of
 c-phycocyanin before
 (Diagram 5) and after
 (Diagram 6) tricalcium
 phosphate chromatography.



C-phycoerythrin samples occurs on storage.

The C-phycoerythrin solution obtained by elution of the main coloured band from the Sephadex column above was freeze-dried, and further examined by Disc Electrophoresis. A sample of the freeze-dried protein was dissolved in a small quantity of water, and the resulting solution centrifuged to remove small quantities of insoluble blue material, presumably protein denatured by the freeze-drying process. The clear solution was mixed in a 1:1 ratio with the Sephadex G-200/sucrose/tris-HCl system (pH 6.7) described by Broome (1963) for the preparation of disc-electrophoresis samples. Two electrophoresis tubes were loaded with this mixture, and electrophoresis (at 200V) carried out for a period of 90 minutes. The tubes were then removed from the apparatus, and one of the gel columns was stained with naphthalene black by immersing it for 20 minutes in a 1 per cent solution of naphthalene black in 7 per cent aqueous acetic acid, and then removing excess dye by immersion in several changes of 7 per cent aqueous acetic acid. The same electrophoresis pattern was evident from both tubes: there was a sharp, intensely coloured band 1.3cm. from the origin, a less sharp and rather less intensely coloured band centred about 1.7 cm. from the origin and about .3cm. deep; and lastly a faint band, possibly split in two, and centred about 2.8cm. from the origin. The red phycoerythrin fluorescence was still clearly visible in the two main bands in the column which had not been stained.

In view of recent work which has been done on the aggregation and disaggregation properties of C-phycoerythrin

[see for example, Berns and Edwards 1965], the three bands described above were tentatively suggested to be respectively the dodecamer, hexamer, and monomer, and the possible splitting of the last band might conceivably be due to the presence of the trimer, although this latter suggestion is purely speculative.

Crystallization of C-phycocyanin.

The procedure used was based on the methods of O'Carra (1962) and Leibo and Jones (1964) and was carried out on a sample which had been stored under 40 per cent ammonium sulphate solution following purification by calcium phosphate chromatography. The protein suspension was centrifuged, and the protein was re-dissolved in distilled water and the solution centrifuged to remove any insoluble material. 40 per cent ammonium sulphate (w/v) was added to this solution over a period of three hours with stirring, the operation being carried out at 0°C in the dark to minimise the possibility of denaturation of the protein. The mixture was kept in the dark at 2°C for 48 hours, and finally centrifuged (1,200g.) for 30 minutes at 0°C. The precipitated protein was dissolved in the absolute minimum quantity of distilled water and the blue, intensely fluorescent solution was dialysed for 48 hours against 0.01M sodium phosphate buffer (pH 6.6) at 2°C, with four changes of buffer. To the dialysed solution 30 per cent ammonium sulphate (w/v) was added and the resultant suspension kept overnight prior to centrifugation at 1,500g. for 40 minutes at 0°C. The supernatant was discarded, and the precipitate was redissolved in the minimum amount of distilled water and the solution dialysed

against 0.01M sodium phosphate buffer (pH 6.6) for 48 hours, with four changes of buffer. To this solution was added 15 per cent (w/v) ammonium sulphate, and the solution was left to stand in the dark room overnight. A small amount of precipitate was obtained on centrifugation at 1,000g. for 30 minutes at 0°C, and this was discarded. The ammonium sulphate concentration of the supernatant was then brought up to 20 per cent, and the solution was left to stand in the dark at 2°C. After some days, an amorphous precipitate had formed; this was removed by centrifugation at 1,500g. for 40 minutes at 0°C and discarded. A further 5 per cent ammonium sulphate (w/v) was added to the supernatant over a period of 2 hours in darkness and the solution allowed to stand for two weeks at 2°C. At this point examination under the microscope revealed the presence of large numbers of needle-shaped, blue crystals of C-phycoerythrin.

Amino acid analysis of C-phycoerythrin.

A sample of C-phycoerythrin which had been purified by Sephadex G-100 chromatography as described earlier and subsequently freeze-dried, was hydrolysed and analysed on the amino acid analyser in the usual way. The results of the analysis are given in Table 6.

TABLE 6. Amino acid Analysis of C-Phycocyanin [cf. Table 3a]

	No. of μ mols in sample	"Rounded off" values [*]	% by weight
Alanine	12.0	38	8.49
Arginine	4.68	15	7.36
Aspartic Acid	8.30	27	9.80
Cysteic Acid	0.28	1	0.54
Glutamic Acid.	7.68	25	10.20
Glycine	7.90	25	4.51
Histidine	0.55	2	0.98
Isoleucine	4.26	13	4.65
Leucine	7.62	25	8.94
Lysine	3.65	12	4.48
Methionine	1.16	3	1.24
Phenylalanine	2.72	9	4.17
Proline	2.68	10	3.06
Serine	6.50	21	5.76
Threonine	5.88	19	6.05
Tyrosine	3.79	11	5.66
Valine.	6.29	21	6.55

* - based on one cystine (\rightarrow cysteic acid) residue per integral unit [Page 69]

Carbohydrate Estimations on C-Phycocyanin.

The method used to estimate the carbohydrate content of C-phycocyanin was the Phenol-Sulphuric Acid method. (Dubois et al., 1956). The method is as follows: The sample (1 ml.) (which should contain between 10 and 70 μ g. carbohydrate) is pipetted into a clean dry test-tube. Aqueous phenol solution (1 ml. 5 per cent) is added, and the mixture shaken. Analar concentrated sulphuric acid (5ml.) is added from a fast flowing burette, such that the jet of acid hits the surface of the liquid directly, to ensure rapid and thorough mixing. Blanks are prepared in the same way, using water (1ml.) in place of the sample. The tubes are agitated during the mixing procedure, and exactly the same mixing procedure is used throughout. Ten minutes after mixing, the tubes are re-shaken and placed in a water-bath at 25-30°C for 20 minutes. The yellow/orange colour produced is stable for several hours; after being allowed to stand for two hours, the optical densities of the solutions are measured. The mean absorbance of the water blanks is subtracted from the sample absorbance and the amount of carbohydrate present determined by reference to a standard curve, using standards containing known amounts of carbohydrate. In the present work, glucose was used as standard, and absorbances were measured on an EEL Colorimeter using a Green Filter (No. 623), or at 487m μ using a Unicam SP500. Determinations in triplicate minimise errors, and permit the exclusion of anomalous results due to contamination e.g. by paper.

C-phycocyanin samples which had been stored under 40

per cent ammonium sulphate solution were used for the carbohydrate estimations. The protein was isolated from the suspension by centrifugation and dissolved in a small quantity of distilled water. Dialysis to remove the last traces of ammonium sulphate was not used since it has been found (Paterson, 1967) that the use of dialysis can lead to anomalous results due to the contamination of the sample by glycerol which is used in the manufacture of the dialysis membrane. Accordingly, the protein solution was desalted by use of a Sephadex G-25 column (20 x 1.5cm.), the small salt molecule being eluted much more rapidly than the large protein molecule. The sample was loaded and eluted with distilled water; by testing the eluate with distilled water it was confirmed that the ammonium sulphate and the protein were eluted separately. The salt-free protein solution was then freeze-dried, and dried in a desiccator over phosphorus pentoxide.

Approximately 10mg. samples of the protein thus prepared were weighed out accurately and hydrolysed for 6 hours at 100°C with 4ml. 2N sulphuric acid in a sealed Pyrex tube. After this period, the tubes were cooled, opened, and the contents of each tube loaded onto a Zeokarb 225 column (12 x 1cm.) in its H⁺ form. This cation exchange resin strongly adsorbs the prosthetic group material from the hydrolysed sample, forming a dark green band at the top of the column. The column was eluted with distilled water, and 20mls. only of eluate were collected; 1ml. of this solution was then used for the carbohydrate estimation by the procedure already outlined. Blanks containing only 4ml.

2N sulphuric acid were subjected to the same treatment, the absorbance reading of the blank being subtracted from that of the sample.

A number of samples of C-phycoerythrin from different harvests of the protein were thus examined: the mean value for the carbohydrate content of C-phycoerythrin was found to be around 1 per cent; the largest value found was 2.1%, the smallest, 0.2%. No attempt was made to identify the individual sugars present.

Small quantities of B-phycoerythrin and B-phycoerythrin, both of which had been isolated from the alga *Rhodospira rubra* by Dr. D.D. Howard, were also available, and although these were possibly impure and almost certainly denatured, they were considered suitable for preliminary investigations of some of the reactions used.

One of the main aims of this research project was to establish, if possible, the nature of the chemical bond which links the tetrapyrrolic chromophore group of C-phycoerythrin to the rest of the protein molecule. A large part of the experimental work to be described under the heading "Degradative Studies" was aimed at solving this problem: it was hoped that it might prove possible to isolate by some means a small chromopeptide - that is, a small peptide with the chromophore group still attached and with the chromophore-protein linkage intact - and that further work on this material might establish the nature of this linkage.

As was mentioned in the Introduction, there is a certain amount of evidence which suggests that the chromophore-protein linkage in certain biliproteins is an

Degradative studies on C-Phycocyanin.

For purposes of comparison, a number of the experiments to be considered under the above heading were carried out not only on C-phycocyanin but also on the protein B-phycoerythrin, which was isolated from the Red alga *Porphyridium cruentum* by Dr. G.M. Paterson. Both proteins were stored under ammonium sulphate solution or as freeze-dried samples until required. The ultra-violet/visible absorption spectrum of aqueous B-phycoerythrin is shown for reference purposes in Diag.7. Small quantities of R-phycocyanin and B-phycoerythrin, both of which had been isolated from the alga *Rhodomenia palmata* by Dr. D.D. Heard, were also available, and although these were possibly impure and almost certainly denatured, they were considered suitable for preliminary investigations of some of the reactions used.

One of the main aims of this research project was to establish, if possible, the nature of the chemical bond which links the tetrapyrrolic chromophore group of C-phycocyanin to the rest of the protein molecule. A large part of the experimental work to be described under the heading "Degradative Studies" was aimed at solving this problem; it was hoped that it might prove possible to isolate by some means a small chromopeptide - that is, a small peptide with the chromophore group still attached and with the chromophore-apoprotein linkage intact - and that further work on this material might establish the nature of this linkage.

As was mentioned in the introduction, there is a certain amount of evidence which suggests that the chromophore-apoprotein linkage in certain biliproteins is an

ester-type linkage, and recent work in this laboratory (Paterson, 1967) has lent support to this view.

Accordingly, a number of the experiments which will be described, were based on the assumption that the linkage is an ester-type linkage through a glutamic acid or aspartic acid ω -carboxyl group to a hydroxy-group on the chromophore molecule.

The degradative work carried out will be described under three separate headings, namely (1) Acidic hydrolyses
(2) Basic hydrolyses
(3) Enzymic hydrolyses.

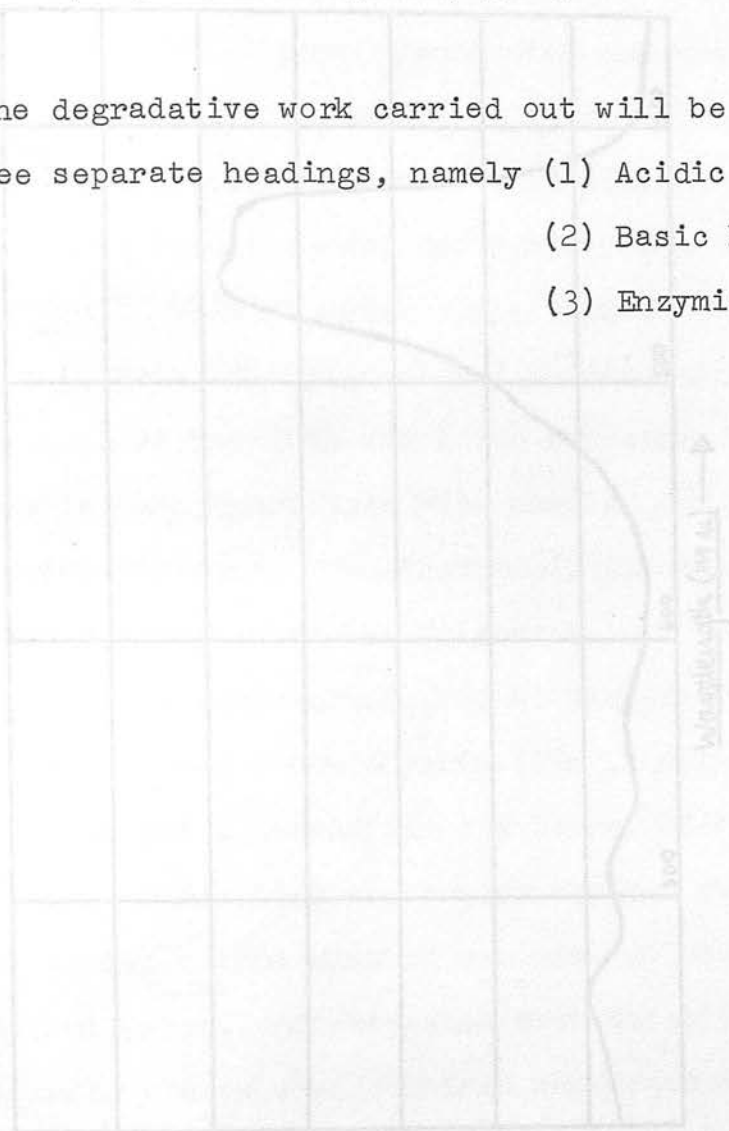


Figure 1 Absorption spectrum of 8-phycocyanin

Acidic Phosphatase

... of acid on C-phosphoryl and other ...
... fairly extensively studied, and the
... in this ...
... In the present
... and no ...

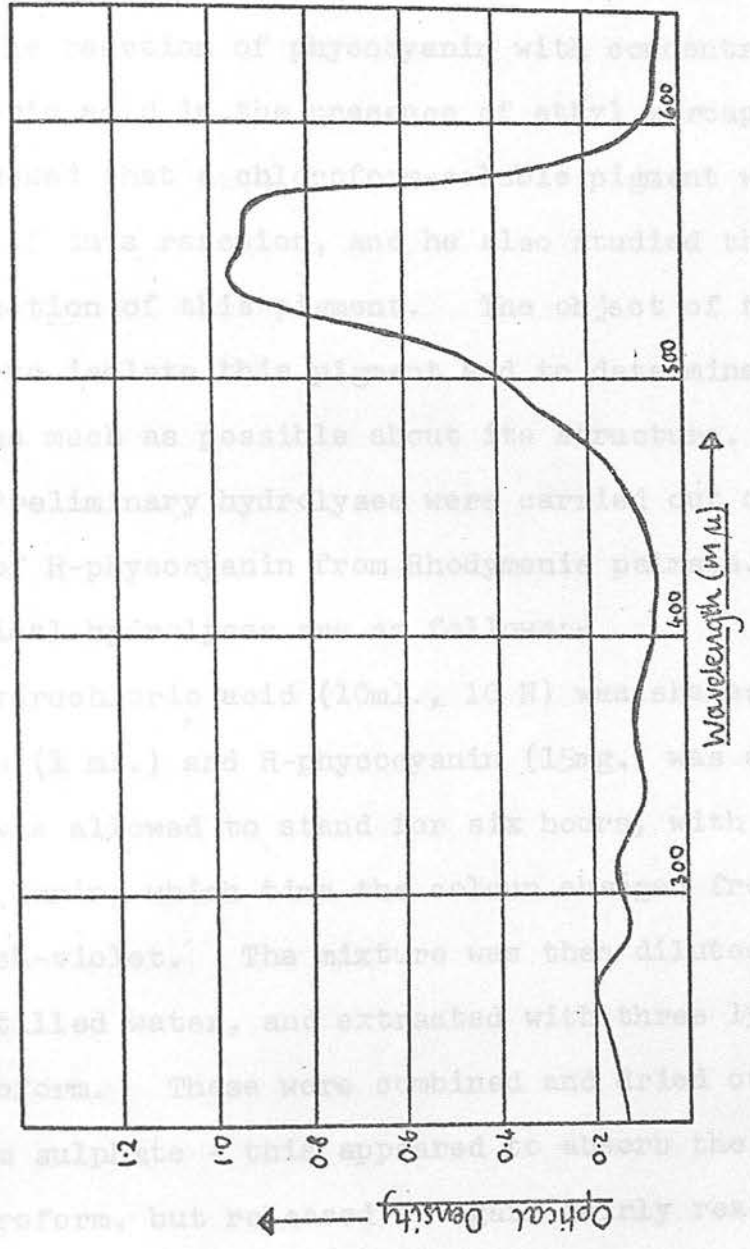


Diagram 7 Absorption spectrum of B-phycoerythrin.

Acidic Hydrolyses.

The effect of acid on C-phycocyanin and other bili-proteins has been fairly extensively studied, and the results and conclusions of workers in this field have already been reviewed in the Introduction. In the present work, attention was concentrated on one particular reaction, namely, the reaction of phycocyanin with concentrated hydrochloric acid in the presence of ethyl mercaptan. O'Carra (1962) showed that a chloroform-soluble pigment was one of the products of this reaction, and he also studied the rate of the production of this pigment. The object of the present work was to isolate this pigment and to determine by spectral methods as much as possible about its structure.

Preliminary hydrolyses were carried out on denatured samples of R-phycocyanin from *Rhodymenia palmata*. Details of a typical hydrolyses are as follows:-

Analar hydrochloric acid (10ml., 10 N) was shaken with ethyl mercaptan (1 ml.) and R-phycocyanin (15mg.) was added. The mixture was allowed to stand for six hours, with occasional shaking, during which time the colour changed from deep-blue to reddish-violet. The mixture was then diluted to 50ml. with distilled water, and extracted with three 15ml. aliquots of chloroform. These were combined and dried over anhydrous magnesium sulphate - this appeared to absorb the pigment from the chloroform, but released it again fairly readily on washing with more dry chloroform. The solution was evaporated to dryness using a rotary evaporator; more chloroform was added, and the mixture re-evaporated in order to remove any residual hydrochloric acid. The solid was dissolved in 1 ml. carbon disulphide, in which it dissolved fairly readily, and

nuclear magnetic resonance, visible, and infrared spectra obtained of the solution. The visible spectrum showed a maximum absorption at $499\text{m}\mu$, with two smaller maxima at around $380\text{m}\mu$ and $580\text{m}\mu$. The infra-red spectrum showed strong absorption at $2,900\text{cm}^{-1}$ with a smaller peak at $2,820\text{cm}^{-1}$, slight absorption - possibly carbonyl - at $1,700\text{cm}^{-1}$ and a sharp, low intensity peak at 770cm^{-1} . The first two peaks and the last suggest the presence of ethyl mercaptan in the sample. The nuclear magnetic resonance spectrum showed a doublet at 8.78 , with a diffuse peak at 9.15 . This again suggested the presence of ethyl mercaptan.

In an attempt to obtain more information from the nuclear magnetic resonance spectrum, the hydrolysis was repeated using 100mg . of phycocyanin, and increasing the quantities of hydrochloric acid and ethyl mercaptan accordingly. The chloroform extract from this hydrolysis gave a visible spectrum differing from that of the first hydrolysis in the shape of the curve, although the maxima occurred in approximately the same places. The nuclear magnetic resonance spectrum yielded no more information than the first spectrum, and it was concluded that it represented a partial spectrum of ethyl mercaptan.

The hydrolysis procedure was repeated, this time using 200mg . of B-phycoerythrin from *Rhodomenia palmata*. The chloroform extract was evaporated down as before, and dried in a high vacuum desiccator, using an oil pump, to free the product from residual ethyl mercaptan. A visible spectrum of this material in chloroform solution was obtained;

this was similar to the previous spectra with the principal maximum at $499\text{m}\mu$, and smaller maxima at 370 and $575\text{m}\mu$. This chloroform solution was evaporated to dryness and the material redissolved in carbon disulphide, giving a solution from which infra-red and nuclear magnetic resonance spectra were obtained. The nuclear magnetic resonance spectrum was similar to the spectra obtained for the phycocyanin hydrolysis product, with absorption in the region $7.3-7.7\tau$. The spectrum of this region was re-run at a higher sensitivity, but the noise level was too high for any significant information to be obtained. To check on the possibility that this absorption might be a carbon-13 absorption associated with the two main peaks, the tetra-methyl silane standard in the sample solution was removed by evaporation, and the spectrum re-run without a standard. A small peak was observed at 9.98τ (previously concealed by the tetra-methyl silane), which could probably be ascribed to carbon-13, and this would be associated with a peak at around 7.58τ . The peak at 9.98τ however was of much smaller intensity than the absorption in the $7.3-7.7\tau$ region, and it was therefore concluded that, although the latter region probably included absorptions due to carbon-13, there were other absorptions present as well.

In order to obtain an infra-red spectrum free of carbon disulphide absorptions, the sample was incorporated in a potassium bromide disc, and the spectrum obtained using this disc. The most satisfactory results were obtained by allowing a few drops of a carbon disulphide solution of the pigment to evaporate in a small mortar, which was then dried

overnight in a vacuum desiccator. A small amount of Analar potassium bromide was then added, and the mixture ground with a pestle, and the disc prepared by the application of pressure. The spectrum thus obtained showed a sharp peak at 2900cm^{-1} with an associated peak at 2840cm^{-1} and further peaks at 1700, 1470 and 1390cm^{-1} . Low intensity absorption at 3400cm^{-1} could possibly be ascribed to N-H vibrations.

The hydrolysis procedure was next applied to 15mg. of purified, freeze-dried C-phycoerythrin. The hydrolysis products were examined by thin layer chromatography on silica-gel-coated microscope slides. The solvent system acetic acid:benzene 1:3 (v/v) led to resolution of the pigment mixture into three components: a purple component ($R_f = 0.12$), a green component ($R_f = 0.08$), and a blue component ($R_f = 0.05$). The latter, blue, component changed slowly to purple on the slide. The bulk of the pigment solution was loaded on to a small (10 x 0.3cm.) silica gel column, and eluted with the same solvent; observation of the column during elution showed that all three coloured bands emitted orange fluorescence. The fast moving, purple, band was collected, and the solution evaporated to dryness. No further use was made of this solution, as it was stored for a long time before it could be used, and its appearance indicated that some deterioration had occurred.

A similar hydrolysis was carried out on 50mg. purified, freeze-dried B-phycoerythrin from *Porphyridium cruentum*; silica gel column chromatography as above gave a diffuse orange band, followed by a sharper purple band - this latter component was eluted, evaporated to dryness, and examined by

nuclear magnetic resonance using carbon disulphide as solvent. In spite of the use of CAT (Computer of Average Transience) which reduces the background noise level, no significant results were obtained, due to insufficient sample being available.

At about this time, the results of Cole et al. (1967) and Crespi et al. (1967) appeared, and no further work in this direction was therefore attempted.

Basic Hydrolyses.

Preliminary studies of the alkaline hydrolysis of biliproteins were carried out on denatured B-phycoerythrin from *Rhodomenia palmata*. B-phycoerythrin (15mg.) was suspended in tris/hydrochloric acid buffer (30ml., pH 8.6, 0.2M), and n-butanol (20ml.) floated on top. The mixture was stirred with a magnetic stirrer overnight, and the resulting pale-green n-butanol solution separated. It was thought at first that this pale green colour might be due to a pigment released from the protein, but examination of the ultra-violet and visible spectra of the solution showed that the colour was in fact due to the presence of small amounts of rivanol (2-ethoxy-6,9-diamino-acridine lactate) which had been used in the preparation of the protein sample, and subsequent experiments showed that rivanol was extracted simply by stirring the protein with water and n-butanol.

The aqueous phase from the above hydrolysis was examined by repeating the experiment using B-phycoerythrin (100mg.) and tris/hydrochloric acid buffer (50ml., pH 8.6, 0.2M), with n-butanol (20ml.). Samples of the aqueous layer were withdrawn at intervals and filtered through Whatman No.42 paper to clarify them. The optical density of each sample was then measured at 500m μ , a sample of unhydrolysed protein solution being placed in the sample beam and the hydrolysate in the reference beam in order to obtain positive values for the optical density. No significant trend could be observed in these results; one possible reason for this is that the pigment first liberated recombines with protein fragments.

The bulk of the aqueous solution was evaporated to small volume, which led to the formation of a green colloidal precipitate, which was separated by centrifugation, and dissolved in aqueous acetic acid (5ml. 20 per cent v/v). This solution was loaded on to a Sephadex G-25 column (20 x 1cm.) and eluted with 20 per cent acetic acid at a flow rate of around 20ml. per hour, and the eluate collected on a turntable. Two green bands were observed, the first being collected after 100 minutes, the second after around 5 hours. These two fractions were evaporated to dryness and hydrolysed overnight with 6N (constant boiling) hydrochloric acid, at 100°C, in a sealed Pyrex test-tube. One-dimensional amino-acid chromatograms were run of each sample, using Whatman No.1 paper, and solvent B, flowing downwards. The first fraction, and hence the higher molecular weight fraction, gave 8 spots on development with ninhydrin, and the second gave 6 spots. It was concluded that a certain degree of fragmentation of the protein had occurred under the conditions used, but that the chromophore had not been cleaved, since both coloured bands still contained a number of amino acids.

Attention was now turned to the effect of high pH on purified C-phyocyanin. Hydrolysis at three different pH values was examined, and the progress of the hydrolysis was followed by observing the rate of disappearance of the absorbance maximum at 615m μ .

(1) At pH 8.4, using sodium phosphate buffer, hydrolysis was found to take several days, and was thus too slow to be studied conveniently.

(2) At pH 10, using sodium carbonate/sodium bicarbonate buffer, hydrolysis was too rapid for further investigation,

being complete within a few seconds of the reagents being mixed.

(3) At pH 9.1, using boric acid/borax buffer, hydrolysis was found to proceed at a suitable rate for study, being about nine-tenths complete, in terms of the initial and terminal values of the 615m μ absorbance, after 400 minutes. A graph of transmittance against log (time) gave a reasonably straight line, indicating that the rate of reaction decreased exponentially with the protein concentration.

The aqueous solution from this pH 9.1 hydrolysis was reduced to small volume by vacuum desiccation over concentrated sulphuric acid. The solution was loaded onto a Sephadex G-25 column (20 x 1cm.), and eluted with ammonium carbonate solution (0.1M), which can be completely removed by evaporation. The eluate was collected in tubes on a turntable at ten-minute intervals, and the absorbance of each tube measured at 280m μ . A pale green band was eluted with the solvent front, and was presumably of high molecular weight; a second band was eluted after 60 minutes.

An attempt was made to confirm the above absorbance readings by the use of trinitrobenzene sulphonic acid, using the method of Satake et al. (1960). 1 ml. of each sample was hydrolysed with sodium hydroxide solution (1 ml. 2N) at 100 $^{\circ}$ to remove ammonium carbonate, and to hydrolyse the peptide material present. The pH was then adjusted to between 7.5 and 8.5 with acetic acid, and trinitrobenzene sulphonic acid solution (1 ml. 0.1 per cent), and sodium bicarbonate solution (1 ml., 4 per cent). added. The mixture was kept in the dark at 40 $^{\circ}$ C for two hours, and then

hydrochloric acid (1 ml. 1N) added. The absorbance at 340m μ was then measured, using a blank prepared by substituting distilled water for sample solution.

No conclusive results could be obtained by this method, even after two recrystallizations of the trinitrobenzene sulphonic acid from water. This was apparently due to the difficulty of removing all the ammonia from the solution.

The R-phycocyanin from *Rhodymenia palmata* which was available was not very soluble in water, presumably due to being largely denatured, but dissolved fairly readily in 0.1N sodium hydroxide solution to give a cloudy green solution. The colour of a sample of this solution was changed to violet on the addition of hydrochloric acid. At pH 6.0 the colour became blue, and this solution gave a blue precipitate on centrifugation. The green alkaline solution from 100mg. phycocyanin gave a green precipitate on careful neutralisation with acetic acid; this precipitate was separated by centrifugation and dissolved in Tris/HCl buffer of pH 8.6, and loaded on to a Sephadex G-25 column (20 x 1cm.). The column was eluted with water; the eluate was collected in tubes on a turntable, and the absorptions at 280m μ of each tube measured. The results were plotted as a function of time. The resulting curve showed a maximum after 50 minutes, corresponding to a cloudy green solution; a second, narrower peak after about 150 minutes corresponding to an olive-green band, and a faint greenish-blue band was eluted after about six hours. Examination of the two main bands by paper electrophoresis, using No. 54 paper, a voltage gradient of

20-22 volts per cm., and solvent I showed that both were complex mixtures of a number of peptides; paper chromatography on Whatman No.1 paper using solvent C gave at least 8 spots, on ninhydrin treatment, from each band, peptide bands (and peptides). Only trypsin, chymotrypsin, and pepsin are sufficiently specific in their action for a controlled degradation of proteins. The other enzymes like subtilisin, trypsin and bacterial proteases are more suitable for further degradation of the larger fractions.

A number of factors control the rates at which these enzymes attack certain peptide bonds. Apart from the spatial arrangement inherent in amino acid sequences the folding of the chain imposes further structural relationships. Many native proteins are resistant to attack by enzymes but are readily digested after denaturation or oxidation. The splitting of certain bonds may modify the behaviour of adjacent susceptible bonds. The cleaved products themselves tend to influence the specificity of an enzyme, leading to unexpected cleavage products.

Although enzymes are more stable at low temperatures digests at 37-40°C allow faster reaction rates. Difficult bonds may be split using the forcing conditions of a high enzyme to substrate ratio. The optimum conditions for the controlled degradation of a protein are best obtained by construction of hydrolysis rate curves.

Three enzymes were utilized in the present work for the degradation of lipoproteins. These were trypsin, pepsin, and chymotrypsin, and a brief description of the properties

Enzymic Hydrolyses.

Proteolytic enzymes, or proteases, are available from a large number of animal, plant, fungal, and bacterial sources. Of the many enzymes capable of splitting internal peptide bonds (endopeptidases), only trypsin, chymotrypsin, and pepsin are sufficiently specific in their action for a controlled degradation of proteins. The other enzymes like subtilisin, mould and bacterial proteases are more suitable for further degradation of the larger fractions.

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Three enzymes were utilised in the present work for the degradation of biliproteins. These were trypsin, pronase, and pepsin, and a brief description of the properties

of each of these will be given before the experimental details are described.

Trypsin. Trypsin is isolated from bovine pancreatic juice. It is normally used in the pH range 7-9, but owing to a strong tendency to autolyse it is not possible to keep the enzyme active for long periods at high pH. The autolysis is retarded considerably in the presence of calcium ions, and it has been suggested (Nord and Bier, 1953) that the metal ion prevents association between two different molecular species. Under conditions in which the enzyme is completely inactivated within six hours, the presence of 0.001M calcium ion leads to a loss of less than five per cent of the activity. The stabilizing effect of calcium and other ions on a number of proteins is a well studied phenomenon (Yon, 1959).

The most valuable property of trypsin is its narrowly restricted specificity which limits cleavage exclusively to bonds linking the carboxyl group of a basic amino acid to the amino group of another amino acid or to the hydroxy group of an alcohol. It has long been known that certain native proteins are resistant to the action of trypsin but that proteolysis takes place readily after denaturation of the protein. On the basis of amino acid analysis it is possible to predict accurately the number of peptides that might be expected to occur in a tryptic digest of a denatured protein.

Pronase. The proteolytic enzyme Pronase, otherwise known as *Streptomyces Griseus* Proteinase, was first isolated by Nomoto et al. (1956). It is the least specific of all known proteolytic enzymes. Its action on protein and peptide

substrates reveals few types of peptide bonds which are totally resistant to hydrolysis under optimal conditions. It is especially useful where extensive cleavage at a variety of bonds is required. The products of the action of Pronase and other similar bacterial proteinases are often quite analogous to the products of partial acid hydrolysis but they are obtained in higher yields than can be expected in acid hydrolysates.

Pepsin. Commercial crystalline pepsin is obtained from gastric juice or gastric mucosa and is a mixture of enzymes. It is fairly stable at pH 5.6, is denatured above pH 6, and is optimally active on synthetic substrates at pH 4 and on proteins at pH 1-2. The enzyme exhibits a very wide specificity, and although experiments with synthetic substrates have shown that a preferential attack is made at bonds involving the amino groups of aromatic amino acids, there are many instances where such bonds in proteins are resistant. Only peptide bonds are attacked, amide and ester bonds being resistant. The specificity of pepsin is enhanced at low enzyme-substrate ratios by limiting the reaction time to around 30 minutes.

Tryptic Hydrolyses.

The trypsin sample used was supplied by the Sigma Chemical Co., St. Louis, Missouri. As it had been stored for some time prior to use, its activity was estimated by two methods. The first method used was that described by Kunitz (1947). 1 gm. Casein was suspended in sodium borate/boric acid buffer (100ml., pH 7.6, 0.2M), which was 0.005M with respect to calcium chloride. The suspension was heated for 15 minutes in a water bath at 35°C, to bring about solution. The solution was found to be slightly cloudy, and was filtered through a plug of glass wool before use.

A solution of the trypsin sample - 1mg. in 50ml. of buffer - was prepared; determinations were carried out in triplicate. 1ml. aliquots of the trypsin solution were pipetted into 15ml. Pyrex tubes, and the tubes placed in a water-bath at 35°C. Casein solution (1 ml.) was pipetted into each tube; the time was noted, and after exactly 20 minutes, aqueous trichloroacetic acid (3ml. 5 per cent) was added to each tube. The contents of each tube were well mixed, and the tubes were removed from the water-bath and allowed to stand for one hour. They were then centrifuged for 20 minutes, and the optical densities at 280m μ measured. Blanks were prepared by mixing one ml. of the casein solution with trichloroacetic acid solution (3ml. 5 per cent), and then adding trypsin solution (1 ml.). The results, obtained by reference to Kunitz's standard curve, show that the trypsin sample used has about 16 per cent of the activity of pure trypsin.

The second method of estimating tryptic activity was less quantitative, being based on the assumption that for every peptide bond cleaved by hydrolysis, a carboxyl group and an amino group are exposed. Measurement of the amount of base required to neutralise these carboxyl groups would then afford an estimate of the extent of the cleavage which had occurred. Casein solution (8ml., as above) was placed in a thermostatted container, at 35°C , and the pH adjusted to 7.6. The pH value was kept constant by means of a pH-stat, made by Radiometer, Copenhagen, Denmark, which adds sufficient acid or alkali to maintain the pH at any selected value; the amount of acid or alkali added is automatically recorded on a chart.

Since trypsin selectively cleaves lysine- and arginine-activated peptide linkages, and casein contains 63.8 and 22.5 moles respectively of lysine and arginine per 10^5 molecular weight units, it can be shown that hydrolysis of 0.08g. casein should liberate $6.9 \cdot 10^{-5}$ moles of carboxylic acid groups. In fact $2.2 \cdot 10^{-5}$ moles of alkali were required for neutralization. This discrepancy was probably largely due to the fact that the release of carboxyl groups is accompanied by the release of amino groups whose effect would be to reduce the amount of alkali required for neutralization.

The results of these experiments suggested that although the trypsin sample was largely deactivated, it probably retained sufficient activity to bring about at least some degree of degradation. Accordingly, purified C-phycocyanin (5mg.) was dissolved in distilled water (8ml.),

and placed in a thermostatted vessel at 35°C . The pH was adjusted to 7.6 by the addition of the requisite amount of sodium hydroxide solution (0.01M). 1 ml. of the 1 per cent trypsin solution was added, and digestion allowed to proceed, the pH being kept at 7.6 by means of the pH-stat as before. After four hours a further 1 ml. of trypsin solution was added, and digestion allowed to proceed until no further uptake of base was observed. It was found that no significant uptake of base occurred unless the protein was first denatured by immersing the solution in a boiling water-bath prior to digestion. A calculation similar to that carried out above for casein showed that about 0.15ml. of 0.01N sodium hydroxide solution should be required for neutralisation of the acidic groups released, and the amount actually used was found to be of this order, but it was not possible to measure accurately so small an amount.

The solution from the tryptic digest was freeze-dried, and dissolved in a small volume of 50 per cent aqueous acetic acid, and the resulting solution was examined by paper chromatography and paper electrophoresis.

Ascending paper chromatography on Whatman No.54 paper using solvent C gave around six ninhydrin-positive spots.

Electrophoresis on No.4 paper for 90 minutes at 20 volts per cm. using buffer I gave one ninhydrin-positive spot only, 0.5cm. on the anode side of the origin. Attempts to combine electrophoresis and chromatography did not prove successful.

The only conclusion which can be drawn from these results is that trypsin does cleave the C-phycoerythrin molecule

to some extent; the nature of the cleavage products remains to be determined.

At this point experiments using trypsin were discontinued since the results of experiments using pronase appeared to be more promising.

The digestion, which seemed to occur readily with the native protein. This method has been shown to be of value in the study of proteins (1963).

A series of the experiments were carried out using pronase rather than trypsin, both for purposes of comparison and also because the former material was more readily available.

Pronase Digest 1. B-phycoerythrin (1 mg.) was dissolved in calcium chloride solution (10 ml., 0.01M); in most cases the protein dissolved initially, but dissolved as the digestion proceeded. The pH was adjusted to 8.5 by the addition of sodium hydroxide solution (0.01M) through a syringe. The experiment was carried out under an atmosphere of nitrogen. The pH was maintained at 8.5 by means of the Radiometer pH-stat. Pronase (2 per cent w/w) was added as 0.05 per cent solution in 0.01M sodium chloride solution, and a further smaller portion of the Pronase solution was added after 1 hour. Fairly rapid

uptake of alkali was observed immediately after the addition of the first portion of the enzyme, indicating that the protein was being digested. The rate of alkali uptake was then slowly decreased. The addition of the second portion of Pronase caused a rapid increase in the rate of alkali uptake. The rate of alkali uptake was then slowly decreased.

Pronase Digestions.

The non-specific proteolytic enzyme Pronase was used by Fujiwara (1960) to digest a tryptic hydrolysate of a biliprotein, but not to digest the intact protein. It was not found necessary to denature the protein before commencing digestion, which seemed to occur readily with the native protein. The method used was similar to that of Marks et al. (1963).

A number of the experiments were carried out using B-phycoerythrin rather than C-phyococyanin, both for purposes of comparison and also because the former material was more readily available.

Pronase Digest 1. B-phycoerythrin (25mg.) was dissolved in calcium chloride solution (10 ml., 0.015M); in fact not all the protein dissolved initially, but dissolved as the digestion proceeded. The pH was adjusted to 8.5 by the addition of sodium hydroxide solution (0.01M) through a micro-burette. The experiment was carried out under an atmosphere of nitrogen; the pH was maintained at 8.5 by means of the Radiometer pH-stat. Pronase (2 per cent w/w) was added as 0.05 per cent solution in 0.015 molar calcium chloride solution, and a further similar portion of the Pronase solution was added after 2 hours. Fairly rapid uptake of alkali was observed immediately after the addition of the first portion of the enzyme, indicating fairly rapid cleavage of the protein; the rate then slowly decreased. The addition of the second portion of Pronase caused a slight rise in the rate of alkali uptake, but the rate rapidly decreased again and after a total hydrolysis

time of eight hours alkali uptake had ceased. 3.1 mls. 0.01M sodium hydroxide solution were found to have been added, and assuming the molecular weight of the B-phycoerythrin under the reaction conditions to be 290,000, then it was calculated that alkali was added at the rate of 360 moles per mole of protein, or that each molecule was cleaved 360 times. Hence the mean molecular weight of the cleavage products is around 800, and in fact this figure is probably high, in view of the conclusions drawn from the results of similar experiments on the tryptic digestion of casein, namely that the liberation of amino groups at the same time as carboxyl groups effectively reduces the alkali titre.

The visible spectrum of the hydrolysis mixture had a main absorption maximum at 580 m μ and other absorptions at 510m μ , 540m μ and 630m μ .

The hydrolysed solution was reduced in volume by rotary evaporation, and loaded onto a Sephadex G-25 column (20 x 1 cm.), and eluted with de-aerated aqueous acetic acid (1 per cent v/v). The eluate was collected on a fraction collector, and the optical densities of the contents of the tubes were measured at 580m μ . The graph obtained by plotting these optical density values as a function of time showed several distinct maxima: the first was eluted from the column after about 30 minutes and corresponded to a pale pink band; the second, which was the most intense band, was eluted from two to three hours after the start, and corresponded to a pink band. After that were eluted a number of low intensity bands, which had no observable

colour. The contents of the tubes containing the main coloured band were combined, reduced to small volume, and repassed through the Sephadex G-25 column, again being eluted with de-aerated 1 per cent aqueous acetic acid. The eluate was collected in the same way as before, and the optical densities of the contents of the tubes measured, this time at 580 and 510m μ . The plot of the optical densities against time showed a maximum after 45 minutes with a distinct shoulder at 75 minutes. The eluate from the column was re-evaporated to small volume and passed through a Sephadex G-15 column (10 x .7cm.) and eluted with 1 per cent aqueous acetic acid. Measurement of the optical densities at 510m μ of the fractions, collected at 4 minute intervals, showed almost complete resolution of the pink material into two bands of roughly equal size, eluted after 12 and 28 minutes respectively. A third peak of low intensity was observed after 44 minutes.

The two main bands were evaporated to dryness and used for amino acid analysis, but no satisfactory results were obtained.

Pronase Digest 2. The experimental details for this experiment are the same as for Pronase Digest 1. The amount of titrant used in this case was found to be 5.2ml. 0.01N sodium hydroxide, which gives a mean molecular weight for the hydrolysis cleavage products of around 400, if allowance is made for the carbohydrate content of the B-phycoerythrin sample used, which had been determined as 17.1 per cent.

The aqueous solution was reduced in volume by evaporation in vacuo over phosphorus pentoxide; the resulting solution was loaded onto a Sephadex G-25 column (20 x 1cm.) and eluted with de-aerated aqueous acetic acid (1 per cent). The eluate was collected on a fraction collector at five-minute intervals. The optical density of each tube was measured at 510 and 580m μ , and these values were plotted as a function of the tube number. A small peak corresponding to a slight pink colour of the sample was observed in tubes 14 to 18; the contents of tubes 40 to 68 were pink, and the portion of the graph corresponding to these tubes showed partial resolution into two components, with the maximum optical density values occurring at tubes 50 and 60. The contents of tubes 47 and 48 were combined, as were those of tubes 65 and 66, and their visible spectra recorded to determine what spectral differences - if any - existed between these two partially resolved components. The two spectra were almost identical, each having a maximum at around 495m μ , but the absorbance of the maximum corresponding to tubes 65 and 66 was of relatively greater intensity, the rest of the two spectra being essentially the same.

The contents of tubes 40 to 60 were combined, again reduced in volume in vacuo over phosphorus pentoxide, and the solution loaded onto a Sephadex G-15 column and eluted as before, the eluate again being collected on a fraction collector, and the optical densities of the tube contents again being measured at 510m μ and 580m μ . The resultant graph was nearly identical with that obtained at the

corresponding stage in Pronase Digest 1, three peaks being clearly defined, although not completely resolved. The contents of the tubes corresponding to each of the three peaks were combined, the three solutions were evaporated to dryness, and each was hydrolysed for amino acid analysis. However, it was not possible to do this as the contents of each hydrolysis tube charred, presumably due to the high carbohydrate content (17.1 per cent) of the B-phycoerythrin sample used.

Pronase Digest 3. Attention was now turned to the effects of Pronase on C-phycoyanin. C-phycoyanin (5mg.) was digested under the same conditions as those used for the first two digests; two portions of Pronase were added in solution, each corresponding to 2 per cent w/w. 1.0ml. 0.01N sodium hydroxide solution was required to maintain the pH at 8.5, which indicates a mean molecular weight for the hydrolysis fragments of 500. A visible spectrum of the hydrolysis mixture showed maxima at 583m μ and 628m μ , the ratio of the respective absorbances being 1.19:1. The solution was freeze-dried and a solution of the freeze-dried material used to spot paper chromatograms and electrophoretograms. However no significant results were obtained, save that cleavage of the protein into several components had occurred.

Pronase Digest 4. The conditions used were the same as those of Pronase Digest 3, save that ammonium carbonate solution containing ammonia replaced sodium hydroxide solution as titrant, the former having the advantage that it is completely volatile. After completion of the hydrolysis, the solution

was reduced in volume on the rotary evaporator at under 40°C , giving a dark green solution. Silica gel thin-layer chromatography using solvent C gave a separation into an orange fluorescent spot at the origin, a red fluorescent spot at around R_f 0.4, and a pale blue fluorescent spot at R_f 0.9 when observed under ultra-violet light; ninhydrin positive material was found at R_f 0.25, with some streaking between that point and the origin.

Pronase Digest 5. The digestion procedure was the same as before, using sodium hydroxide solution as titrant.

C-phycoerythrin (15.4mg.) were hydrolysed; the digest was reduced in volume on the rotary evaporator, loaded onto a Sephadex G-25 column (20 x 1cm.) and eluted with de-aerated aqueous acetic acid (1 per cent), which gave a pale green band. Further elution was carried out using 10 per cent and 50 per cent aqueous acetic acid; these two fractions were combined - solution (2) - and this and the 1 per cent fraction - solution (1) - were reduced in volume by rotary evaporation.

Thin-layer chromatography as in Pronase Digest 4 of solution (1) gave a pale yellow colour with ninhydrin at around R_f 0.35; solution (2) showed a blue fluorescent spot under ultra-violet illumination at R_f 0.9, and some ninhydrin-positive material at around R_f 0.4. High voltage electrophoresis was carried out on Whatman No.4 paper for 30 minutes at 3.5kV (70 volts per cm.), using buffer H. Solution (1) showed only one yellow band with green fluorescence about 1cm. to the cathode side of the origin

line; solution (2) gave two bands with fairly intense blue fluorescence at 1.2cm. and 2cm., bands 2a and 2b respectively, from the origin line on the anode side. Two other pale fluorescent bands were observed close to the origin line. Bands 1, 2a and 2b were cut out of the electrophoresis paper and eluted with 20 per cent aqueous acetic acid and evaporated to small volume. Examination of the bands by thin-layer and paper chromatography provided no useful information; each gave rise to a number of ninhydrin positive spots.

Samples 2a and 2b were examined by cellulose thin-layer two-dimensional chromatography using solvent system D. Both samples appeared to have very similar compositions, and assuming that the spots on the chromatograms were due to free amino acids, there was a preponderance of the neutral amino acids leucine, iso-leucine, valine, alanine and glycine with smaller amounts of the basic and acidic amino acids. The two samples were hydrolysed in the usual manner for amino acid analysis and the two-dimensional chromatography repeated. The amino acid patterns obtained in each case were similar to those obtained before hydrolysis, although there was a considerable amount of streaking at that point on the chromatogram where the chromophore group or its breakdown products would be expected to occur.

The hydrolysates were examined also by thin-layer electrophoresis, using buffer F at 16 volts per cm. for 40 minutes, to determine whether any basic or acidic amino acids were present. In fact none were found by this method.

Investigations based on the assumption of a chromophore protein ester type linkage through a carboxyl group of a glutamic or aspartic residue required the preparation of a number of model compounds, namely, the mono-hydroxy acids formed by reduction of one or other of the carboxyl groups of glutamic and aspartic acids, and the diols formed by the reduction of both carboxyl groups.

(1) Homoserine, formed by the reduction of the ω -carboxyl group of aspartic acid, was obtained from the Sigma Chemical Company.

(2) 2-amino-3-hydroxybutyric acid, theoretically derived from aspartic acid by the reduction of its α -carboxyl group, was prepared from asparagine by the method of Bruckner et al. (1959). Asparagine was treated with benzoyl chloride to give N-benzoyl-asparagine which was esterified with diazomethane to give N-benzoyl-asparagine-methyl ester. This was reduced with lithium borohydride to give N-benzoyl-2-amino-3-hydroxy-asparagine, which was hydrolysed to 2-amino-3-hydroxy butyric acid.

(3) 2-amino-1,4-dihydroxy butane, the compound derived theoretically by the reduction of both carboxyl groups of aspartic acid, was prepared from aspartic acid via its diethyl ester hydrochloride which was prepared using ethanolic hydrochloric acid by the method of Angier et al. (1950). This was converted to the free base by treatment with diethylamine in benzene, and the free diester was reduced with lithium aluminium hydride to 2-amino-1,4-dihydroxy-butane, purified by distillation under reduced pressure. (Karrer and Portmann, 1948).

(4) 1-amino-4-hydroxyvaleric acid, derived theoretically from glutamic acid by reduction of the ω -carboxyl group, and otherwise known as δ -hydroxy-norvaline, was prepared from glutamic acid, which was first reacted with toluene-p-sulphonyl chloride to give N-p-toluenesulphonyl-glutamic acid (Rudinger et al, 1959). Treatment of this with phosphorus trichloride in chloroform solution gave the cyclic product 1-p-toluene-pyrrolid-S-one-carboxylic acid monohydrate, which was converted to the anhydrous form by azeotropic distillation in benzene, and then to its lithium salt by treatment with lithium methoxide in methanol. Reduction of this salt with lithium borohydride in tetrahydrofuran gave 4-hydroxy-1-tosyl-aminovaleric acid, and finally, treatment of this with sodium in liquid ammonia gave the required product (Goodman and Felix, 1964).

(5) 3-amino-4-hydroxyvaleric acid, theoretically derived from glutamic acid by reduction of the α -carboxyl group, was prepared by the same method as that described for the corresponding derivative of aspartic acid, starting from glutamine.

(6) 2-amino-1,5-dihydroxypentane, theoretically derived from glutamic acid by the reduction of both carboxyl groups, was prepared from glutamic acid by the same method as that described above for the preparation of the corresponding derivative of aspartic acid.

Pronase Digest 6. C-phycoerythrin (15mg.) was hydrolysed in the usual manner, except that portions of solid Pronase (2 per cent w/w) were added instead of a Pronase solution. After 8 hours' digestion, the solution was reduced in volume

on the rotary evaporator, giving a deep purple solution. This solution was loaded onto a Sephadex G-25 column (20 x 1cm.) and eluted with 1 per cent aqueous acetic acid, giving a purple band - solution 7-1. Elution overnight with more of the same solvent gave solution 7-2. 50 per cent aqueous acetic acid eluted a green band - solution 7-3; and further washing with this solvent gave solution 7-4. All four solutions were reduced in volume by rotary evaporation and examined by high voltage electrophoresis, using Whatman No.3MM paper, and buffer H; electrophoresis was carried out for 30 minutes at 70 volts per cm. Sample 7-1 gave one greenish-brown band with green fluorescence about 1cm. from the origin on the negative side; sample 7-2 showed four fluorescent bands, one at the origin, one on the negative side of the origin and 1.1cm. from it, and two on the positive side of the origin, 1 cm. (a) and 2.9cm.(b) distant from it. Sample 7-3 showed slight fluorescence at the origin, and at 0.4cm. from it on the positive side; and sample 7-4 showed slight fluorescence at the origin, and about 0.5cm. away from it on either side. Attention was concentrated on the bands (a) and (b) from sample 7-2, since their elution characteristics indicated relatively low molecular weight, and the intensity of their fluorescence suggested the presence of chromophore residues. The two bands were eluted from the paper with 20 per cent aqueous acetic acid, and the two solutions evaporated to dryness on a rotary evaporator and re-dissolved in small amounts of water. The two bands appeared to be homogeneous on further high voltage electrophoresis. The colour of the fluorescence

appeared to be dependent on the nature of the support medium; spots of either solution on cellulose thin layers had green fluorescence, while on silica gel thin layers the fluorescence was white.

Fraction 2a was evaporated to dryness and thoroughly dried in vacuo over phosphorus pentoxide, and reduced by refluxing overnight with a solution of lithium borohydride in tetra-hydrofuran. This solution was prepared by stirring an excess of lithium borohydride with tetrahydrofuran which had been distilled from calcium hydride, and removing solid material by centrifugation. Measurement of the amount of hydrogen liberated from this solution in the presence of cobaltous chloride catalyst showed that the solution was approximately 0.4M with respect to borohydride (Elliott et al, 1952.) After reduction, excess lithium borohydride was destroyed by the addition of water, followed by 5N hydrochloric acid, to the reaction mixture, which was then evaporated to dryness. The residue was hydrolysed with 6N hydrochloric acid in the usual manner, and the resulting solution evaporated to dryness; the evaporation was repeated twice with water. The residue contained the amino acids resulting from the hydrolysis and also large quantities of salts, mainly lithium chloride and lithium borate, and in order to carry out accurate amino acid analyses of the hydrolysate, it was necessary to find a method of removing these salts. The first method tried was high voltage electrophoresis: a solution containing a number of amino acids and sodium chloride was subjected to paper electro-

phoresis using buffer F on No. 3MM paper; 70 volts per cm. being applied for 20 minutes. The relative movement of the sodium and chloride ions from sodium chloride was observed by the pH change as indicated by the colour changes undergone by methyl red; the sodium was also detected by spotting with zinc uranyl acetate. It therefore appeared possible to isolate the neutral amino acids both from salt contaminants and also from the acidic and basic amino acids, thus simplifying the resulting chromatography. The method however proved unsatisfactory when used with an actual hydrolysate, as treatment of the electrophoretogram by spraying first with 2N hydrochloric acid followed by turmeric solution showed that borate was not removed by this method, and this was thought to be the cause of the streaking which occurred on two-dimensional thin-layer chromatography on cellulose plates, using solvent system D.

This de-salting procedure was further studied by reducing 50mg. of casein with lithium borohydride in tetrahydrofuran as above. After removal of the solvents by evaporation in the usual way, the residue was hydrolysed with 6N hydrochloric acid, and the residue treated with methanolic hydrochloric acid to remove boron as volatile methyl borate, the solvent being removed by rotary evaporation. This treatment was carried out twice. The solid was stored overnight in a vacuum desiccator containing sodium hydroxide pellets, and then subjected to high voltage paper electrophoresis as described above. The area containing the neutral amino acids was located by ninhydrin development of a strip, and this area was eluted with 20 per cent aqueous

acetic acid, the solution being evaporated to dryness and stored in a sodium hydroxide desiccator. The residue was dissolved in a small quantity of water and examined by thin-layer chromatography; development with ninhydrin showed that streaking had occurred near the origin, but treatment with the turmeric reagent showed that this was not due to the presence of borate salts. Hydrolysis of a sample of casein led to a chromatogram free from streaking in that region, and the possibility therefore arose that the streaking might arise from the chromophore group of the biliprotein molecule, and not as a product of the reduction process. The streaking found on the reduced casein chromatogram might be due to incomplete hydrolysis resulting in peptide material being present as well as amino acids.

Pronase Digest 7. During the carbohydrate analyses carried out on C-phycoerythrin it was observed that some coloured material derived from the chromophore group was strongly adsorbed onto the Zeokarb 225 column used, and further study was made of this phenomenon to see if any useful information could be obtained. B-phycoerythrin (25mg.) was dissolved in sodium borate/boric acid buffer (50ml., pH 8.5, 0.1M) and solid Pronase (1mg.) added. The mixture was incubated at 30°C under toluene for 24 hours with occasional agitation, and further Pronase (1mg.) added. Incubation was continued for a further 24 hours, and the volume of the solution reduced to about 5ml. by rotary evaporation. The solution was loaded onto a Zeokarb 226 column, forming a pink band at the top. The column was eluted successively with 1 per

cent, 10 per cent, 20 per cent, and 50 per cent aqueous acetic acid, giving respectively solutions 7-1, 7-2, 7-3 and 7-4. The coloured material was eluted as two bands by the 50 per cent acetic acid; these were collected separately as 7-4a and 7-4b. All five solutions were reduced to small volume by rotary evaporation, and examined by thin-layer silica gel chromatography, using solvent C.

Solution 7-1 showed a ninhydrin positive spot at $R_f 0.3$. Solution 7-2 showed a pale green area at around $R_f 0.5$, with green fluorescence; there was also green fluorescence at around $R_f 0.9$. Solution 7-3 showed a pale yellow-coloured streak extending from $R_f 0.4$ - 0.8 , with pale blue fluorescence. Solution 7-4a showed two pink spots at $R_f 0.2$ and 0.4 , with orange fluorescence; there was also a small amount of white fluorescence near the origin. Solution 7-4b showed one pink band at $R_f 0.3$, with orange fluorescence.

The five solutions were also examined by thin-layer cellulose chromatography, using solvent C. Solution 7-1 showed a number of ninhydrin-positive spots; solution 7-2 showed one slightly fluorescent spot at $R_f 0.5$, which was ninhydrin-positive. Solutions 7-3 and 7-4a both gave fluorescent streaks, extending from the origin to around $R_f 0.3$, and solution 7-4b gave two orange-fluorescent spots at $R_f 0.1$ and 0.3 . On a silica gel thin-layer plate, using acetic acid:benzene (1:3 v/v), solution 7-4b gave a purple spot at $R_f 0.3$ with slight streaking between the spot and the origin.

Solutions 7-1, 7-4a and 7-4b were next examined by

thin-layer electrophoresis using cellulose thin-layer plates and buffer G. Electrophoresis was carried out for 40 minutes at 25 volts per cm. Solution 7-1 gave two ninhydrin spots on the negative side of the origin and 1.8 and 1.1 cm. distant from it; there was also one white fluorescent spot at the origin, and another 1.0cm. from it on the positive side. Bands 7-4a and 7-4b both gave spots with pale orange fluorescence about 1.0cm. on the negative side of the origin, and white fluorescent spots about 1.0cm. on the positive spots.

Bands 7-4a and 7-4b were next examined by thin-layer cellulose chromatography, using the solvent system D. Chromatograms were run both before and after hydrolysis with 6N hydrochloric acid. The two chromatograms of the unhydrolysed samples were very similar: a preponderance of the neutral amino acids leucine, isoleucine, phenylalanine, valine, alanine and serine was observed, together with a number of other spots, probably due to small peptides. The amino acid pattern after hydrolysis was similar, and there was a certain amount of streaking, probably caused by breakdown products of the chromophore group.

Pronase Digest 8. B-phycoerythrin (100mg.) was hydrolysed with Pronase using the same method as in Pronase Digest 6. The hydrolysis mixture was centrifuged to remove a small amount of insoluble material, and the resulting solution was reduced to small volume on the rotary evaporator. The solution was loaded onto a Sephadex G-100 column (15 x 2cm.), and eluted successively with 250ml. portions of 1 per cent, 5 per cent and 50 per cent aqueous acetic acid. Of the

four solutions thus obtained, the first three were evaporated to dryness on the rotary evaporator and stored in a desiccator. The 50 per cent acetic acid wash was evaporated to dryness and stored for some weeks; after this time an aqueous solution of this material was found to exhibit strong fluorescence when spotted on filter paper, and it was accordingly studied by electrophoresis under various conditions.

Electrophoresis on 3MM paper at pH 4.0 using buffer I for 90 minutes at 20 volts per cm. gave one pale fluorescent band at the origin, and two blue fluorescent bands on the positive side of the origin and 0.7 and 1.2cm. distant from it. Development with ninhydrin gave three bands on the negative side of the origin, 7.2, 5.0 and 1.0cm. distant from it, and one band on the positive side of the origin line, 3.0cm. from it.

High voltage electrophoresis on 3MM paper using 5 per cent aqueous acetic acid for 45 minutes at 70 volts per cm. was next used. A pale yellow band with green fluorescence was observed 1 cm. from the origin line on the negative side (band a); a band with intense blue fluorescence was observed just on the positive side of the origin (band b); and a diffuse band with blue fluorescence was observed around 1cm. from the origin on the positive side (band c). Bands a, b and c were eluted from the paper using 20 per cent aqueous acetic acid; the three solutions were reduced in volume and re-examined by high voltage paper electrophoresis under the same conditions as above. All three bands gave a blue fluorescent band just on the positive side

of the origin; sample a gave three pale fluorescent bands on the negative side of the origin, as well as a number of ninhydrin positive spots; samples b and c both gave a number of ninhydrin positive spots. The three blue fluorescent bands, now referred to respectively as d, e and f, were eluted from the paper with 20 per cent aqueous acetic acid, evaporated to dryness on the rotary evaporator, and each was taken up in a small volume of water. Solutions d, e and f were then examined by paper electrophoresis using buffer G on Whatman No.1 paper for 2 hours at 20 volts per cm. All three samples gave identical results - each gave two fluorescent bands: one on the negative side, 0.5cm. from the origin, and one on the positive side, 1.5cm. from the origin; the latter band was of greater intensity. All of sample d was subjected to electrophoresis in this way, and this intensely fluorescent band was eluted with 20 per cent aqueous acetic acid; the solution was evaporated, and the residue reduced with lithium borohydride in tetrahydrofuran in the manner described in Pronase Digest 6. After removal of the solvents, the salty material remaining was hydrolysed with 6N hydrochloric acid for amino-acid analysis. Unfortunately, the tube exploded while in the oven, and the sample was thus lost. The process was therefore repeated with sample e, and after hydrolysis, the acid was removed by rotary evaporation. Desalting was accomplished firstly by evaporation twice with methanolic hydrochloric acid to remove boric acid, followed by high voltage electrophoresis, using the procedure described in Pronase Digest 6. Two pale yellow bands were obtained; one was centred around 2 cm. from the origin line on the

negative side and showed pale blue fluorescence under ultra-violet light; the other was centred on the origin line. The narrow region in between these two was found to be ninhydrin-positive, and was carefully cut from the paper and eluted with 20 per cent aqueous acetic acid. The solution was reduced to dryness and stored in a desiccator over sodium hydroxide, prior to analysis on the Amino Acid Analyser.

The chromatogram obtained from this sample showed considerable salt contamination, and no quantitative results could be obtained. However aspartic acid, threonine, serine, glycine, alanine and valine, and smaller amounts of glutamic acid, leucine, and isoleucine were present; there also appeared to be present a small quantity of homoserine, one of the reduction products of aspartic acid.

The above results showed that a more efficient method of desalting reduction mixtures was required. Electro-dialysis, described in the section on General Techniques, was eventually found to be the solution to this problem; the desalting process was carried out before hydrolysis, since it was thought that relatively large peptides were less likely to be lost during desalting than amino acids. Accordingly, sample f was subjected to the same process as e above, save that following hydrolysis, the solvents were removed by evaporation, and the salty material was dissolved in water. The aqueous solution was desalted and then evaporated to dryness, and the residue hydrolysed and its amino acid content determined on the Amino Acid Analyser. The chromatogram obtained was free from any traces of salt

contamination. The results of this analysis are shown in Table 7; the most striking features of the results are the relatively large amounts of glycine and alanine. There did not appear to be any significant amount of homoserine present.

The 1 per cent acetic acid wash from the Sephadex G-100 column was next examined. The sample was dissolved in a small volume of sodium phosphate buffer (0.1M, pH 7.0), and loaded onto a Sephadex G-100 column (20 x 0.7cm.) which had been calibrated for molecular weight determination by use of three substances of known molecular weight, namely trypsin inhibitor, bovine serum albumin, and blue dextran. On elution with sodium phosphate buffer (0.1M, pH 7.0), the molecular weight of the sample was found to be approximately 13,000.

Pronase Digest 9. B-phycoerythrin (40mg.) was digested with Pronase by the method of Pronase Digest 6. After digestion was complete, the volume of the hydrolysis mixture was reduced by rotary evaporation, and loaded onto a Sephadex G-10 column (1.5 x 20cm.). A broad pink band - 9-1 - was eluted with distilled water, and the column was washed successively with 200ml. portions of 1 per cent, 5 per cent, 25 per cent and 50 per cent aqueous acetic acid. Each of the bands thus eluted - bands 9-2, 9-3, 9-4 and 9-5 respectively, was evaporated to dryness and stored in a desiccator. Solution 9-1 was freeze-dried, to give 54.7mg., presumably containing small quantities of salt, of dark red material. This dark red material was dissolved in a small quantity of water and examined by paper electrophoresis on 3MM paper using buffer G. Electrophoresis

was continued for 2 hours at 15 volts per cm. The bulk of the coloured material was found to remain at the origin, with a green fluorescent band immediately adjacent to it on the negative side, and a blue fluorescent band immediately adjacent to it on the positive side. A number of ninhydrin-positive spots were also observed.

High voltage electrophoresis was next used on 3MM paper, using buffer G. Electrophoresis was carried out for 30 minutes at 70 volts per cm. The red material was found to extend from about 0.5cm. on the negative side of the origin to 3.5cm. on the positive side. Immediately adjacent to it on the negative side was a green fluorescent band, and adjacent to it on the positive side were two blue fluorescent bands, about 1 cm. apart. There were also a number of ninhydrin positive spots.

The above high voltage electrophoresis was repeated with the bulk of the red material, and that portion of the resulting electrophoretogram between 0.5cm. and 2cm. on the positive side of the origin was cut out, and the pink material eluted from it with 20 per cent aqueous acetic acid. The solution was evaporated to dryness and reduced, desalted and hydrolysed by the procedure described under Pronase Digest 8. The hydrolysate was then examined using the Amino Acid Analyser. The results of the analysis are given in Table 8; the most striking feature was the large amount of glycine, and there were also relatively large amounts of proline and alanine, as well as a small amount of homoserine.

The material obtained by the evaporation of the 50 per cent acetic acid wash - sample 9-5 - was treated with phenyl

isothiocyanate by the method of Gottschalk and Murphy (1961). The dried material was dissolved in a mixture of 1.0ml. water:1.0ml. N-allylpiperidine pyridine buffer (1gm. N-allylpiperidine in 39ml. pyridine, pH adjusted to 9.0 with N acetic acid). 0.1ml. phenylisothiocyanate was added, and the mixture then heated in a glass-stoppered test-tube at 40°C for 1 hour with occasional shaking. The cooled mixture was extracted seven times with equal volumes of benzene, and the aqueous layer then concentrated to dryness in a desiccator. The dry residue was then reduced, desalted and hydrolysed by the procedure described in Pronase Digest 8, and finally analysed on the Amino Acid Analyser in the usual manner. The results are shown in Table 9; they show large amounts of glycine, proline, and alanine, and also glutamic acid, lysine, and arginine.

Residue	Sample 1	Sample 2	Sample 3
Alanine	0.28 (1)	0.25 (1)	0.33 (1)
Aspartic acid	0.14 (2)	0.51 (3)	0.68 (2)
Glutamic acid	-	0.65 (2)	1.74 (4)
Glycine	0.20 (1)	-	-
Proline	0.20 (1)	-	-
Serine	0.70 (3)	2.34 (8)	1.31 (4)
Threonine	1.27 (5)	0.67 (2)	1.03 (3)
Tyrosine	0.48 (2)	0.63 (2)	0.12 (1)
Valine	0.16 (1)	-	0.23 (1)
Unknown	0.46 (2)	0.48 (2)	2.91 (3)

The first set of figures in each row is the number of peaks of each residue in the sample and the figures in parentheses are the relative amounts.

TABLE 7. Amino acid composition of "fluorescent" material from Pronase digestion of B-phycoerythrin [Page 110].

TABLE 8. Amino acid composition of "pink" material from Pronase digestion of B-phycoerythrin [Page 111].

TABLE 9. Amino acid composition of "fluorescent" material (as in Table 7) treated with phenyl isothiocyanate and reduced. [Page 112].

	Table 7	Table 8	Table 9.
Alanine	1.22 (5)	1.98 (7)	2.92 (9)
Arginine	-	0.87 (3)	2.70 (9)
Aspartic Acid	0.74 (3)	0.63 (2)	0.82 (2)
Glutamic Acid	1.70 (7)	0.93 (3)	2.50 (8)
Glycine	2.34 (10)	5.39 (18)	7.55 (25)
Histidine	-	trace	trace
Isoleucine	0.25 (1)	0.25 (1)	0.33 (1)
Leucine	0.44 (2)	0.51 (2)	0.68 (2)
Lysine	-	0.45 (2)	1.74 (6)
Methionine	0.20 (1)	-	-
Phenylalanine	0.20 (1)	-	-
Proline	0.70 (3)	2.34 (8)	3.31 (11)
Serine	1.27 (5)	0.67 (2)	1.03 (3)
Threonine	0.48 (2)	0.43 (2)	0.43 (1)
Tyrosine	0.16 (1)	-	0.25 (1)
Valine	0.44 (2)	0.48 (2)	0.61 (2)

The first set of figures in each case is the number of μ -moles of each amino acid in the sample used; the figures in parentheses are "rounded-off" values.

Pepsin Digestions.

The method used for the peptic digestion of C-phyococyanin and B-phycoerythrin was basically that of Lemberg (1928).

Pepsin Digest 1. B-phycoerythrin which had been stored under 30 per cent (w/v) ammonium sulphate solution was separated by centrifugation, dissolved in distilled water, and any remaining ammonium sulphate removed by dialysis overnight against running tap water. The protein solution was then freeze-dried, and 230mg. of the freeze-dried material were dissolved in hydrochloric acid (10mls. n/10) containing crystalline pepsin (60mg.), and the mixture incubated at 30°C for 24 hours. After this time, a further 10ml. of hydrochloric acid containing 60mg. pepsin were added and the mixture was incubated for a further 24 hours. The hydrolysis mixture was then centrifuged to remove any insoluble material, and the clear solution was extracted several times with n-amyl alcohol, a total volume of 80mls. being used. Due to a tendency of the two solvents to form an emulsion, centrifugation was used to separate the two layers. A visible spectrum of the organic layer, which showed orange fluorescence somewhat reminiscent of the native biliprotein, gave a main maximum at 550m μ with a slight shoulder at 495m μ , and a broad absorption at around 370m μ . The aqueous layer was treated with five volumes of absolute alcohol and placed overnight in the refrigerator; the pepsin content was precipitated as a colloidal mass which was removed by centrifugation; the visible spectrum of the resulting solution showed a maximum at 554m μ with a

shoulder at 500m μ ; the addition of ammonia led to a shift of this maximum to 591m μ with shoulders at 550 and 510m μ , and a new maximum appeared at 322m μ .

The organic layer was divided into two portions. One was evaporated to dryness by rotary evaporation using the oil-pump, since the boiling-point of n-amyl alcohol is 138°C, and the residue was hydrolysed with 6N hydrochloric acid in the usual way. The hydrolysate was then analysed on the Amino Acid Analyser. The results are given in Table 10. Large amounts of alanine, valine, leucine and isoleucine were found to be present, and it is of significance that these are the amino acids whose solubility in n-amyl alcohol would be expected to be greatest. It therefore seemed to be the case that the large amounts of these amino acids were due to the liberation of these free amino acids or small peptides containing them by the action of pepsin on the protein, and that quantitative analysis of the coloured peptide would require the removal of such contaminants.

The second portion of the amyl alcohol layer was reduced to dryness as before, taken up in a small volume of ethanol and studied in a variety of ways with a view to purifying the coloured material.

Cellulose thin-layer chromatography, using solvent C gave two brownish-purple spots at R_F 0.8 and 0.9; the presence or absence of ninhydrin-positive material could not be determined owing to the similarity between the colour of the spots and the colour of ninhydrin spots.

The sample was next examined by paper electrophoresis on Whatman No.1 paper; buffer G was used and electrophoresis

was continued for 30 minutes at 20 volts per cm. A green fluorescent band and a yellow fluorescent band were observed on the negative side of the origin, respectively 1.1 and 2.3cm. distant from it; a blue fluorescent band was observed on the positive side of the origin line and 0.8cm. from it. There was also some ninhydrin-positive material at the origin.

Cellulose thin-layer electrophoresis was next tried using buffer K; electrophoresis was carried out for 1 hour at 25 volts per cm. Three fluorescent bands were observed: a green band 5.0cm. from the origin on the negative side; a yellow band on the same side of the origin, and 1.0cm. from it; and an orange band just on the positive side of the origin line. A number of ninhydrin-positive bands were found on the negative side of the origin.

An attempt was made to purify this material by high voltage paper electrophoresis, using buffer K and Whatman 3MM paper; electrophoresis was carried out at 100 volts per cm. for 30 minutes. One diffuse pink band was observed between 4.5 and 7.0cm. from the origin line on its negative side. The purity of this band was further studied by eluting it in three portions - that between 4.5 and 5.3cm. from the origin, that between 5.3 and 6.1cm., and that between 6.1 and 7.0cm. The three solutions were each reduced to small volume by rotary evaporation, and the three samples examined by cellulose thin layer electrophoresis, using the pH 2.2 buffer described above. All three samples were found to give rise to a number of ninhydrin-positive bands and it was concluded that high voltage electrophoresis was not a satisfactory purification procedure.

Attention was now turned to the aqueous phase; the volume of this was reduced by rotary evaporation to about one-tenth, to remove the ethanol which had been used to precipitate the pepsin. The resulting pink solution was then subjected to fractional precipitation with ammonium sulphate. An ammonium sulphate concentration of 30 per cent w/v led to the formation of a pink precipitate which was removed by centrifugation; the concentration of ammonium sulphate was increased to 35 per cent w/v, but no further precipitation was observed. A concentration of 40 per cent w/v led to the precipitation of the remainder of the pink material, and this again was separated by centrifugation. The two fractions thus obtained were each dissolved in a small quantity of water, and purified by Disc Electrophoresis, using the same buffer and gel systems as were used in the homogeneity studies on C-phyococyanin. Both samples gave the same electrophoresis pattern: a diffuse pale pink band was observed about 1.5cm. from the origin; there was a narrow pale pink band at about 3.1cm. from the origin, with an intense narrow purplish/pink band at about 3.4cm., and immediately in front a pale yellow band. The intense bands derived from the 30 and 40 per cent ammonium sulphate precipitates were each eluted from their respective gel columns by cutting the column appropriately with a razor blade and eluting the coloured material with water. The eluate derived from the 30 per cent precipitate was dialysed overnight against running distilled water using a micro-dialysis apparatus (Exogen Ltd., 972 Dumbarton Road, Glasgow), to remove the

glycine which contaminated the solution, being present in the buffer used in the Disc Electrophoresis process. The solution was then evaporated to dryness and hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results of the analyses are given in Table 11; the glycine figure is extremely high indicating that removal of the glycine was not complete.

The eluate derived from the 40 per cent ammonium sulphate precipitate was evaporated to dryness and stored in a desiccator.

Pepsin Digest 2. The Pepsin digestion was carried out using the same procedure as in Pepsin Digest 1, but using C-phycoerythrin (80mg.) in place of B-phycoerythrin, and reducing the amounts of pepsin and hydrochloric acid in proportion. The amyl alcohol layer was evaporated to dryness by rotary evaporation using the oil-pump, and the blue residue was dissolved in a few drops of ethanol. Thin-layer silica gel chromatography of this solution using benzene:ethanol (50:50 v/v) as solvent gave two spots, one green at R_F 0.5, and one dark green at R_F 0.7. A small silica gel column (10 x 0.3cm.) was prepared, and a solution of the sample in ethanol loaded on to the column. Elution with benzene gave a dark green band, although it is possible that this band may have been eluted by the ethanol used for loading the sample. This band was collected and hydrolysed for amino acid analysis in the usual way. Only small peaks were obtained on this chromatogram due to

the small amount of material available. The results are given in Table 12. Further elution of the column with 10 per cent ethanol/benzene gave a very pale green band. 50 per cent ethanol/benzene gave a dark green band, with some green material being left at the top of the column.

Pepsin Digest 3. The procedure of Pepsin Digest 2 was repeated using C-phycoerythrin (50mg.); the amyl alcohol layer was divided into two portions. The first fraction was reduced to dryness using the oil pump, and the residue was dissolved in a few drops of ethanol. The solution was applied to a cellulose thin layer chromatography plate and developed upwards with solvent C. The coloured material was found to migrate at the solvent front, and development of the remainder of the chromatogram with ninhydrin gave a number of ninhydrin positive bands, with three in particular, of R_f values 0.25, 0.47 and 0.53 being specially intense. These were tentatively ascribed to alanine, isoleucine and leucine respectively. The coloured material was eluted from the chromatogram by scraping off the relevant area of the chromatogram, and extracting it with a small volume of the chromatographic solvent. The solution was evaporated to dryness by rotary evaporation, and hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results are shown in Table 13a. The amounts of valine, leucine and isoleucine were found to be rather large.

The second portion of the amyl alcohol layer was reduced to dryness using the rotary evaporator and oil pump, and the residue was refluxed with methanol overnight by the

TABLE 10 Amino acid composition of amyl alcohol extract of Pepsin digest of B-phycoerythrin. [Page 114]

TABLE 11. Amino acid composition of aqueous phase from Pepsin digest of B-phycoerythrin. [Page 117]

TABLE 12. Amino acid composition of amyl alcohol extract of Pepsin digest of C-phyocyanin; purified by silica gel chromatography. [Page 118]

	Table 10	Table 11	Table 12
Alanine	7.51 (15)	1.20 (4)	0.54 (6)
Arginine	0.75 (1.5)	-	trace
Aspartic Acid	4.96 (10)	2.19 (7)	0.47 (5)
Cysteic Acid	-	0.98 (3)	-
Glutamic Acid	5.15 (10)	1.85 (6)	0.63 (7)
Glycine	5.60 (11)	very high	1.03 (11)
Histidine	0.30 (1)	-	trace
Isoleucine	7.86 (16)	0.83 (3)	0.77 (8)
Leucine	14.92 (30)	0.94 (3)	1.19 (13)
Lysine	0.94 (2)	-	trace
Methionine	0.30 (1)	trace	-
Phenylalanine	5.95 (12)	1.29 (4)	0.35 (4)
Proline	0.99 (2)	0.52 (1.5)	trace
Serine	4.09 (8)	1.93 (6)	0.65 (7)
Threonine	3.51 (7)	0.63 (2)	0.27 (3)
Tyrosine	4.83 (10)	1.22 (4)	0.27 (3)
Valine	7.50 (15)	0.75 (2)	0.69 (7)

The first set of figures in each column represents the number of amino acids in each sample; the figures in parentheses are "rounded-off" values.

method of Crespi et al. (1967), to determine whether or not it was possible by this method to isolate the chromophore free of amino acid residues, as is the case with the intact protein. The residue was refluxed overnight with 90 per cent (v/v) aqueous methanol, and the resulting blue solution centrifuged to remove the solid residue, and evaporated down to dryness on the rotary evaporator. The residue was dissolved in a few drops of ethanol and the solution applied to a cellulose thin-layer chromatography plate, which was developed upwards with the same solvent as above. The coloured material which was light brown in colour was again found to migrate with the solvent front; two fairly intense ninhydrin-positive spots had R_f values 0.05 and 0.09, and there was some faint ninhydrin colour between R_f 0.2 and 0.4. The coloured material was eluted from the chromatogram using the same procedure as above; the solution was evaporated to dryness, and hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results are shown in Table 13b, and these show a marked drop in the amounts of valine, isoleucine and leucine.

Pepsin Digest 4. The procedure used was the same as that used in the previous Pepsin digests, C-phycoerythrin (130mg.) being digested with the appropriate amounts of pepsin and N/10 hydrochloric acid. The digest was extracted as before with n-amyl alcohol, and the solution obtained was evaporated to dryness using the rotary evaporator and oil pump. The residue was dissolved in a small quantity of ethanol, and the coloured material, which appeared to be

TABLE 13. (a.) Amino acid composition of amyl alcohol extract of Pepsin digest of C-phycoerythrin, purified by cellulose thin layer chromatography.

(b.) as above, treated with methanol [Pages 118-9].

(c.) Amino acid composition of "phycoerythrin," prepared by methanolysis of C-phycoerythrin [Page 122]

	Table 13a	Table 13b	Table 13c
Alanine	2.84 (4)	2.26 (3)	2.44 (6)
Arginine	0.21 (0.3)	0.61 (0.8)	-
Aspartic Acid	2.30 (3)	2.17 (3)	2.60 (6)
Cysteic Acid	0.71 (1)	0.35 (0.5)	0.37 (1)
Glutamic Acid	2.99 (4)	3.33 (4)	3.05 (8)
Glycine	4.23 (6)	4.39 (6)	3.82 (10)
Histidine	trace	0.31 (0.5)	-
Isoleucine	8.56 (12)	2.42 (3)	1.09 (3)
Leucine	12.00 (17)	3.65 (4)	1.95 (5)
Lysine	0.60 (1)	0.83 (1)	-
Methionine	trace	-	-
Phenylalanine	4.60 (6)	1.22 (2)	0.85 (2)
Proline	2.64 (4)	0.95 (1)	-
Serine	2.63 (4)	2.25 (3)	2.38 (6)
Threonine	2.52 (4)	1.73 (3)	1.38 (4)
Tyrosine	1.98 (3)	0.51 (0.8)	0.53 (1.5)
Valine	7.22 (10)	2.14 (3)	1.62 (4)

The first set of figures in each column represents the number of μ -moles of each amino acid in the sample; the figures in parentheses are "rounded-off" values.

rather more green in colour than the corresponding material from Pepsin Digest 3, was purified by cellulose thin-layer chromatography as in Pepsin Digest 3, and eluted using a small volume of the chromatographic solvent. The solution thus obtained was centrifuged to remove the cellulose powder, and the clear solution thus obtained was evaporated to dryness. One half of the residue was hydrolysed and analysed on the Amino Acid Analyser in the usual way; the results are given in Table 14a. The other half was dried in vacuo over phosphorus pentoxide and reduced with lithium borohydride in tetrahydrofuran by the method described in Pronase Digest 6. The mixture was then desalted, and hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results are given in Table 14b. The marked differences between these last two analyses were tentatively ascribed to the loss of small (probably acidic) peptides and amino acids during the electrodialysis procedure.

Pepsin Digest 5. The procedure of previous Pepsin digests was followed exactly using freeze-dried C-phycocyanin (50mg.). The residue obtained on evaporation of the amyl alcohol was dissolved in ethanol (10ml.), and a 2 ml. aliquot of this solution was evaporated to dryness and hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results of this analysis are given in Table 15a.

A second 2 ml. aliquot of the above peptide solution was evaporated to dryness and reduced with lithium borohydride; for this reduction commercial grade lithium

TABLE 14. Amino acid composition of amyl alcohol extract of Pepsin Digest 4 of C-phycoerythrin

(a) before reduction.

(b) after reduction. [Page 120]

	Table 14a.	Table 14b.
Alanine	8.90 (10)	3.07 (16)
Arginine	*	*
Aspartic Acid	7.56 (8)	1.54 (8)
Cysteic Acid	-	-
Glutamic Acid.	7.36 (8)	2.36 (12)
Glycine	10.23 (12)	8.16 (43)
Histidine	*	*
Isoleucine	9.95 (11)	0.51 (3)
Leucine	17.9 (20)	0.94 (5)
Lysine	*	*
Methionine	-	-
Phenylalanine	6.36 (7)	0.42 (2)
Proline	7.74 (8)	3.24 (17)
Serine	6.54 (7)	1.35 (7)
Threonine	7.20 (8)	0.69 (4)
Tyrosine	3.68 (4)	trace
Valine	9.41 (10)	0.92 (5)

The first set of figures in each column represents the number of μ -moles of each amino acid in the sample; the figures in parentheses are "rounded-off" values.

* the basic amino acid contents of these samples were not determined.

borohydride was extracted with sodium-dried ether, and the solution was centrifuged to remove insoluble material; evaporation of the solution thus obtained gave a crystalline product (Wilcox, 1967). 10mg. of this purified material was dissolved in tetrahydrofuran (20ml.) which had been dried by distillation from lithium aluminium hydride, and the peptide sample was heated under reflux overnight with this solution. The solution was then treated with a small volume of water, and sufficient hydrochloric acid (5N) was added to neutralise any remaining borohydride. The solution was evaporated to dryness on the rotary evaporator, and boric acid was removed by evaporating to dryness twice with methanolic hydrochloric acid. The residue, containing a small amount of lithium chloride, was hydrolysed and analysed on the Amino Acid Analyser in the usual way. The results of this analysis are given in Table 15b; these suggest that reduction had not taken place, and accordingly the reaction was repeated using the same reaction conditions but with double the amount of lithium borohydride. The results are given in Table 15c.

For purposes of comparison, C-phycoerythrin was methanolysed by the method of Crespi et al. (1967). C-phycoerythrin (20mg.) was heated under reflux overnight with 90 per cent aqueous methanol. The solution was evaporated to small volume, extracted with petrol ether (40-60°), and centrifuged to remove insoluble material. The solution was extracted several times with chloroform, and the chloroform solution was evaporated to dryness. The blue residue was dissolved in a small volume of ethanol

TABLE 15. Amino acid composition of amyl alcohol extract of Pepsin Digest 5 of C-phycoerythrin

(a) before reduction.

(b) after reduction with 10 parts lithium borohydride

(c) after reduction with 20 parts lithium borohydride.

[Page 121]

	Table 15a	Table 15b	Table 15c
Alanine	6.75 (4)	2.10 (4)	2.68 (4)
Arginine	*	*	*
Aspartic Acid	6.38 (4)	2.32 (5)	2.82 (4)
Cysteic Acid	-	-	-
Glutamic Acid	7.72 (5)	2.82 (5)	3.30 (5)
Glycine	11.0 (7)	4.31 (8)	4.81 (7)
Histidine	*	*	*
Isoleucine	16.8 (10)	4.91 (10)	7.09 (11)
Leucine	23.9 (14)	6.80 (14)	9.15 (14)
Lysine	*	*	*
Methionine	-	-	-
Phenylalanine	9.40 (6)	2.84 (6)	3.57 (6)
Proline	5.20 (3)	1.80 (4)	2.66 (4)
Serine	7.81 (5)	2.98 (6)	3.08 (5)
Threonine	6.74 (4)	2.01 (4)	2.50 (4)
Tyrosine	6.50 (4)	1.81 (4)	2.22 (3)
Valine	15.0 (9)	4.52 (9)	6.81 (10)

* - the basic amino acid contents of these samples were not determined.

The first set of figures in each column represents the number of μ -moles of each amino-acid in the sample; the figures in parentheses are "rounded-off" values.

and applied to a cellulose thin-layer plate in the manner described for Peptic Digests. The plate was developed by ascending chromatography with solvent C; the coloured material behaved similarly to the coloured material obtained on peptic digestion, being eluted at the solvent front. A number of ninhydrin-positive bands were observed close to the origin. The coloured material was eluted from the chromatogram using more of the solvent, and the solution was evaporated to dryness on the rotary evaporator in the usual way; the residue was hydrolysed for amino acid analysis in the usual way. Analysis was carried out by two dimensional chromatography on cellulose thin-layer plates, using solvent system D; most of the commonly occurring amino acids were present. The hydrolysis was therefore repeated using 50mg. C-phycocyanin; the chloroform extract was this time purified by the method of Crespi et al. (1967): the residue was dissolved in chloroform containing 2.5 per cent v/v methanol (2ml.), filtered and evaporated to dryness. The residue was redissolved in the chloroform-methanol mixture (1ml.), and precipitated by the addition of 7 volumes of petrol ether (40-60°). The solid material was separated by centrifugation, and washed with more petrol ether. The solid was then dried, and hydrolysed and analysed on the amino acid analyser in the usual way. The results, which are shown in Table 13, show that the "phycocyanobilin" thus purified contains about 2 per cent by weight of peptide material, assuming a recovery of approximately 2 per cent by weight of chromophore from the protein.

Reduction of C-phycoerythrin.

The final experiment was somewhat speculative, being based on the observation that freeze-dried C-phycoerythrin appeared to form a suspension in tetrahydrofuran, and accordingly an attempt was made to reduce this suspension using lithium borohydride by the method previously described. The reduction mixture was desalted by distillation with methanolic hydrochloric acid to remove boric acid followed by electrodialysis, and then hydrolysed and analysed on the amino acid analyser in the usual way. The results are shown in Table 16.

Aspartic Acid	0.91	(5)
Glutamic Acid	0.12	(5)
Glutamine	0.94	(5)
Glycine	1.69	(9)
Histidine	trace	
Isoleucine	0.33	(2)
Leucine	0.56	(3)
Lysine	0.19	(1)
Methionine	—	
Protein	trace	
Proline	0.69	(6)
Serine	0.70	(6)
Threonine	0.66	(2)
Tyrosine	trace	
Valine	0.51	(3)

The first set of figures represents the number of residues of each amino acid in the sample; the figures in parentheses are "rounded-off" values.

TABLE 16.

Amino Acid Composition of C-phycocyanin suspended in tetrahydrofuran and reduced with lithium borohydride [Page 123].

	Table 16
Alanine	0.99 (5)
Arginine	0.11 (0.5)
Aspartic Acid	0.91 (5)
Cysteic Acid	0.12 (0.5)
Glutamic Acid	0.94 (5)
Glycine	1.69 (9)
Histidine	trace
Isoleucine	0.38 (2)
Leucine	0.54 (3)
Lysine	0.19 (1)
Methionine	-
Phenylalanine	trace
Proline	0.69 (4)
Serine	0.70 (4)
Threonine	0.44 (2)
Tyrosine	trace
Valine	0.51 (3)

The first set of figures represents the number of μ -moles of each amino acid in the sample; the figures in parenthesis are "rounded-off" values.

DISCUSSION

Culture of Anabaena Cylindrica. The blue-green alga *Anabaena cylindrica* was cultured in the medium of Allen and Arnon (1955), a current of air containing 5 per cent carbon dioxide being passed through the medium. Certain problems connected with sterilisation of the culture medium and vessels were encountered initially; lack of technique in the inoculation of the sterilised vessels resulted in the growth of large quantities of white mould which although not apparently harmful tended to swamp the growth of the alga. Another possible source of contamination lay in the necessity to autoclave the medium in two portions to avoid precipitation of phosphates, the mixing process being a hazardous one as far as the risk of contamination is concerned. The first attempt to solve this problem - the replacement of phosphate in the medium with glycerophosphate - was not successful, as the resulting medium proved to be barely capable of sustaining algal growth. The second attempt - the use of ultra filtration to sterilise the culture medium as an alternative to autoclaving it - proved a success in that no precipitation of phosphates was observed and there was no evidence of contamination of the medium. The growth rate of the alga was rather slow; Allen and Arnon (1955) reported 7-8 grams dry weight of *Anabaena cylindrica* per litre in 5-6 days, whereas in the present work approximately 1 gram dry weight per litre was obtained after four weeks. Although the amounts of protein produced proved sufficient, larger amounts would have been welcome. The reason for the slow growth rate was not determined; presumably some deficiency in the medium or in the illumination was responsible.

Extraction and Purification of C-Phycocyanin. C-phycocyanin was extracted from freshly harvested cells of *Anabaena cylindrica*. Ultrasonication proved to be the most effective method of liberating the protein from the cells; mechanical methods, such as grinding with sand or repeated freezing and thawing proved to be slow and inefficient. Prior to purification of the crude protein solution it was necessary to remove chlorophyll and other soluble cell fragments; this was done by precipitating the crude protein by the addition of 40 per cent w/v ammonium sulphate; the precipitate was then extracted with water, when it was found that the bulk of the cell fragmentation products did not redissolve. The solution was then centrifuged and filtered through a short column of unsedimented celite to remove the small amount of debris remaining in suspension which would otherwise have blocked the top of the calcium phosphate column used for the purification of the protein. The calcium phosphate chromatography showed that the biliprotein from *Anabaena cylindrica* was almost entirely C-phycocyanin, with a small amount of allophycocyanin. The "special ratio" (see page 11) of the phycocyanin thus purified was generally in the range 3.3 - 4.0; O'hEocha and Raftery (1959) considered that a biliprotein with a spectral ratio greater than 4 was of high purity. Further purification was carried on a sample of protein by chromatography on a column of Sephadex G-100; the protein proved to be virtually homogeneous with respect to this process, but there was little improvement in the spectral ratio. This was probably because the sample used had been stored in the freeze-dried state, and some deterioration may have occurred. Denaturation studies on

the biliproteins (Tanford, 1964; O'Carra, 1965) indicate that denaturation leads to a loss of fluorescence and a decrease in the high wavelength absorptions; and it was therefore concluded that the protein, although chromatographically homogeneous, was slightly denatured. The protein as purified by calcium triphosphate chromatography was considered sufficiently pure for the degradative studies which were carried out.

Examination of the protein by Disc Electrophoresis at pH 6.7 leads to the appearance of three bands; these were ascribed to the dodecamer, the hexamer and the monomer. This is in general agreement with the results of Scott and Berns (1965), although in view of the notorious sensitivity of the C-phycoerythrin system to buffer composition, the latter band might possibly be the trimer rather than the monomer.

Crystallisation of C-phycoerythrin was achieved on samples which had been stored under 40 per cent w/v ammonium sulphate solution; purification of these samples was carried out by fractional precipitation with ammonium sulphate, and the spectral ratio of the protein thus purified was found to be 4.4. This suggests that fractional precipitation is a more effective method of purifying biliproteins than Sephadex column chromatography. This purified protein was dissolved in phosphate buffer of pH 6.6; this pH was chosen rather than the iso-electric point - pH 4.5 - to allow for the decrease in pH which occurs on the addition of ammonium sulphate. In fact the final pH of the solution after the addition of 25 per cent w/v ammonium sulphate was found to be around 5.5. The crystals obtained on allowing this

solution to stand were needle-shaped; Fujiwara (1955) reported that C-phycocyanin crystallised either as needles or prisms, while Hattori and Fujita (1959) reported that phycocyanin from *Tolypothrix tenuis* crystallised as thin platelets. The pH at which crystallisation is carried out appears to influence the shape of biliprotein crystals; this effect has been studied by Bouillene-Walrand and Delarge (1937).

The carbohydrate content of C-phycocyanin from *Anabaena cylindrica* was investigated by acidic hydrolysis followed by application of the phenol-sulphuric acid method (Dubois et al., 1956). The protein sample used was desalted by Sephadex column chromatography rather than by dialysis, as Paterson (1967) showed that small amounts of glycerol from the dialysis tubing led to anomalous results for the carbohydrate content. The results obtained show that C-phycocyanin contains approximately 1.0 per cent carbohydrate. These results are in agreement with those of other workers who have found that biliproteins in general appear to be glycoproteins with relatively low (1-5 per cent) carbohydrate contents (Fujiwara, 1960, 1961; Sasaki and Tsuchiya, 1961) and although no figures have been published for C-phycocyanin from *Anabaena cylindrica*, the values obtained in the present work seem reasonable.

Amino Acid Analysis. A sample of freeze-dried C-phycocyanin was subjected to amino acid analysis, and the results are given in Table 6; although no results have been published for C-phycocyanin from *Anabaena cylindrica*, the figures obtained are in substantial agreement with those for C-phycocyanins from a number of other sources (Table 3b) (O'Reilly and Berns, 1963),

and they are included in Table 3b for purposes of comparison.

The molecular weight calculated on the basis of these figures, based on one cystine or two histidine residues per molecular weight unit, is approximately 31,500, assuming that there are two phycocyanobilin residues per unit (Berns, Scott and O'Reilly, 1964). O'Reilly and Berns (1963) reported the minimum molecular weights of a number of C-phycocyanins to be in the region 28,000-32,000.

Degradative studies: Acidic hydrolyses. Examination of the effect of acid on C-phycocyanin was restricted to the system devised by O'Carra (1962), in which the biliprotein is hydrolysed with 1.0N hydrochloric acid in the presence of ethyl mercaptan, which combines with the pigment released by the acid to give a chloroform-soluble product. It was hoped that study of the nuclear resonance spectrum of this product might enable its structure to be determined and that from this structure it might be possible to deduce the nature of the bond linking the chromophore to the apoprotein in the intact protein molecule. Initial experiments using R-phycocyanin from *Rhodomenia palmata* showed that the pigment was highly contaminated with ethyl mercaptan, but it was found that this could be removed by evaporation in high vacuo. Silica-gel chromatography of the pigment obtained by a similar hydrolysis of C-phycocyanin showed that there were at least three coloured components present, and the pigment obtained on hydrolysing B-phycoerythrin behaved similarly. The major component from a B-phycoerythrin hydrolysis was isolated and its nuclear magnetic resonance spectrum obtained; even by use of a CAT (Computer of Average Transients) however, which averages out

the noise level, there was insufficient sample available to allow any significant results to be obtained, and since there was insufficient protein available to permit the preparation of the required amounts of pigment, this work was discontinued.

Basic Hydrolyses. Heard (1965) found that hydrolysis of R-phyococyanin from *Rhodymenia palmata* at pH 8.6 in the presence of n-butanol led to the appearance of a green colouration in the organic layer, and it was thought that this colour might be due to the release of a pigment from the protein. Study of the spectral properties of this material however showed that the colour was caused by the presence of small quantities of Rivanol, (2-ethoxy-6, 9-diamino-acridine lactate) which had been used for fractionation of the biliprotein mixture from *Rhodymenia palmata* (see Fujiwara, 1955). The difficulty of removing the last traces of Rivanol from protein samples presents a severe restriction to the usefulness of this method of fractionating biliprotein mixtures.

The basic hydrolysis of C-phyococyanin at pH 8.6, as followed by the magnitude of the absorbance at 615 m μ , was found to be 90 per cent complete after seven hours; the rate of hydrolysis was found to decrease exponentially with the protein concentration. Fractionation by Sephadex column chromatography of this hydrolysate and of that obtained by hydrolysing R-phyococyanin with decinormal sodium hydroxide solution gave in each case a number of coloured components; further examination of each of these components by chromatography and electrophoresis showed that each was a complex mixture.

The results of these basic hydrolyses are in general agreement with the findings of Paterson (1967) who noted the formation of a number of chromopeptides by the action of

alkali on B-phycoerythrin. The elution characteristics of the various coloured fractions from the Sephadex columns suggest that they are of fairly high molecular weight; this view is supported by the results of amino acid analyses of the fractions which showed that each contained a number of amino acid residues. There is no evidence to suggest that the chromophore group is cleaved from the protein, although the possibility of cleavage of the chromophore followed by recombination with protein fragments cannot be ruled out. Chapman et al. (1968, in press) claim that the ease of cleavage of phycocyanobilin with potassium hydroxide solution suggests an ester-type linkage, but the reaction conditions they used are not at present known.

Enzymic Hydrolyses. Proteolytic enzymes were found to be the most effective means of cleaving the phycocyanin molecule. Of the three enzymes used - Trypsin, Pronase and Pepsin - Trypsin was found to be the least effective. The degree of cleavage produced by Trypsin digestion, as measured by the uptake of base, was in qualitative agreement with the predicted value based on the number of lysine and arginine residues, Trypsin being specific for peptide bonds activated by these two residues. Examination of the hydrolysates by chromatography and electrophoresis confirmed that cleavage of the protein molecule had occurred, but there was no evidence of cleavage of the chromophore group.

Pronase was found to bring about a much greater degree of cleavage than Trypsin; this is to be expected in view of its lack of specificity. On the basis of the alkali uptake during digestion of B-phycoerythrin or C-phycocyanin, the mean molecular weight of the fragments produced was calculated to

be around 500. Fractionation of hydrolysates on Sephadex columns showed that a number of chromopeptides was produced; the molecular weight of the largest chromopeptide produced by Pronase digestion of B-phycoerythrin was shown to have a molecular weight of around 13,000 by measurement of its rate of elution from a column of Sephadex G-100 which had been calibrated for molecular weight determination by the use of substances of known molecular weight. The elution pattern from the Sephadex column, as followed by the changes in the absorbances of the eluate at fixed wavelengths, was found to be similar to that obtained by Crespi et al. (1968) who used three enzymes in succession (trypsin, chymotrypsin and carboxypeptidase A) to bring about degradation of the protein, and this suggests that the breakdown of the protein may follow a similar course in each case. Examination of the various fractions from the Sephadex column by a variety of chromatographic and electrophoretic procedures showed that each gave rise to a number of ninhydrin-positive spots; these must presumably be due to small peptides or free amino acids. The fraction of greatest interest was that eluted from the Sephadex columns by 50 per cent aqueous acetic acid; the slow elution of this material from the column indicates low molecular weight coupled with a degree of aromatic character, suggesting a relatively small chromopeptide. Paterson (1967) showed that the material thus isolated from B-phycoerythrin both by the action of Pronase and by the action of base possessed the properties which would be expected of a chromopeptide. This material was found to display strong fluorescence under ultra-violet illumination; a sample was

subjected to amino acid analysis after purification by high voltage electrophoresis, and the results are shown in Table 7. Most of the commonly occurring amino acids were found to be present with glutamic acid and glycine being present in the largest quantities. The treatment of peptides with phenyl isothiocyanate has the effect of increasing their solubilities in organic solvent (Gottschalk and Murphy, 1961); a sample of fluorescent material thus treated was reduced with lithium borohydride in tetrahydrofuran, desalted by electrodialysis followed by distillation with methanolic hydrochloric acid, and hydrolysed; the resulting amino acid analysis (Table 9) is similar to that of the unreduced material but with increased amounts of glycine and proline. The increase in the amount of proline is especially interesting since one of the possible products of the reduction of glutamic acid, 2-amino-4-hydroxyvaleric acid, would be readily converted to proline under the strongly acidic conditions used to hydrolyse the reduced sample. Since lithium borohydride is a specific reducing agent for ester groups, these results therefore suggest an ester-type linkage between the ω -carboxyl group of glutamic acid and a hydroxy group on the chromophore. This point will be discussed at a later stage.

The fluorescent material was also observed in the larger molecular weight fractions to some extent, and the amounts of this material appeared to increase if the fractions were allowed to stand; the possibility therefore arises that the fluorescent material is at least in part a product of atmospheric oxidation.

One further experiment was based on the fact that ion-

exchange resins have a strong affinity for aromatic entities, and it had previously been observed during the carbohydrate analysis of the protein that the chromophore-containing material was strongly retained at the top of the ion-exchange column. A sample of B-phycoerythrin was digested with Pronase and loaded onto a column of Zeo-karb 226, a strongly acidic ion-exchange resin; the coloured material was found to be eluted as two bands by 50 per cent aqueous acetic acid. The strong adsorption of this coloured material on the ion exchange resin strongly suggested presence of an aromatic entity; the amounts of phenylalanine and tyrosine did not appear to be sufficiently large to explain this behaviour, and it was therefore concluded that the tetrapyrrolic chromophore was present with a fairly high degree of unsaturation and thus displaying some aromatic character. An interesting point which arises is that such aromatic character would imply coplanarity of the molecule, and it has been suggested that the fluorescence of the native protein is due to the chromophore groups being forced by the configuration of the native protein to assume a rigid co-planar structure (Lavorel and Moniot, 1962). Thin layer chromatography showed that the compositions of the two bands were very similar, and that both were fairly complex mixtures; the neutral amino acids leucine, isoleucine, valine, alanine and serine appeared to be present, and a number of other spots - corresponding presumably to small peptides - were also observed. After hydrolysis, the amino acid composition was found to be rather similar, suggesting that the coloured bands contained a number of free amino acids. The high proportion of the neutral amino acids

leucine, isoleucine and valine provides evidence for a hydrocarbon-like sheath associated with the chromophore, and this point will be discussed at a later stage.

Pepsin digestions were carried out by the method of Lemberg (1928). n-Amyl alcohol extraction of such a digest of B-phycoerythrin gave a pink solution which displayed orange fluorescence similar to that of aqueous solutions of the native protein. The absorption maximum of this solution - 550 m μ - is close to the two absorption maxima of the native protein - 542 and 562 m μ - suggesting that the chromophore is present in a state close to that of the native chromophore. Amino acid analysis of this coloured material (see Table 10) showed a preponderance of alanine, valine, leucine and isoleucine, significantly those amino acids whose solubilities in organic solvents are greatest. It was concluded that free amino acids or small peptides were released by the action of pepsin on the protein and that these were extracted by amyl alcohol along with the coloured material, those with hydrocarbon chains being extracted to the greatest extent. The aqueous phase was also analysed; after fractional precipitation with ammonium sulphate and Disc electrophoresis, the analysis shown in Table 11 was obtained: this shows a preponderance of those amino acids whose solubilities in water are greater than their solubilities in organic solvents - for example, aspartic and glutamic acids.

Pepsin digestion of C-phycoerythrin led to a dark green-coloured organic phase; purification by cellulose thin-layer chromatography was found to be more satisfactory than silica gel chromatography. The former method permitted the removal

of a number of ninhydrin-positive components from the coloured material. An analysis of the purified material is shown in Table 13a; the amounts of leucine, isoleucine and valine are still large.

A sample of the purified material was methanolysed by the method of Crespi et al. (1967); since methanolysis of the intact protein by this method had led to the release of an apparently amino acid-free chromophore, it was anticipated that similar treatment of this coloured material would also lead to release of the chromophore. In fact this was not found to be the case; thin layer chromatography of the methanolysis product confirmed that further breakdown of the coloured material had occurred as demonstrated by the appearance of a number of new ninhydrin-positive spots on thin layer chromatography, but amino acid analysis of the coloured material - which was now brown in colour, suggesting some change in the conjugation - showed that there were still a number of amino-acid residues present; the results are shown in Table 13b. The amounts of leucine, isoleucine and valine are seen to have been fairly markedly reduced, again suggesting the possibility that the chromophore is enveloped in a hydrocarbon-like region of the molecule; this has been suggested by O'hEocha (1963) and by Crespi et al. (1968). The mechanism of the release of the pigment by methanolysis, either as an amino acid-free pigment or with a peptide chain attached, remains obscure.

In view of the evidence which was presented in the Introduction, it seems probable that the chromophore is attached to the apoprotein by ester-type bonds. O'hEocha (1963) and O'Carra et al. (1964) have suggested that these

bonds link the propionic acid side chains on the chromophore molecule to hydroxy groups on serine or threonine residues in the protein chain. However, since the carboxyl groups of the pigments isolated from biliproteins on methanolysis were not esterified (O'Carra and O'hEocha, 1966), the release of the pigments cannot involve methanolysis of such bonds. The results of the present work suggest that, as in the native protein, the chromophore in the pigment obtained by peptic digestion appears to be attached by both covalent and non-covalent interactions (see e.g. O'hEocha and O'Carra, 1961; Lavorel and Moniot, 1962), and that the effect of methanolysis is to cleave that portion of the molecule - the hydrocarbon-like sheath - which is only weakly attached, leaving the chromophore attached probably by an ester-type linkage to a peptide chain; glycine, leucine and glutamic acid were found to be the most abundant amino-acids in this chain (see Table 13b). Crespi et al. (1968) claimed, on the basis of somewhat tenuous evidence, that the peptide chain which remained attached to phycocyanobilin after successive treatment of C-phycocyanin with trypsin, chymotrypsin and carboxypeptidase A, consisted of serine, glycine, alanine, aspartic acid and leucine.

The effect of reduction by lithium borohydride in tetrahydrofuran solution on the coloured material obtained by peptic digestion was next examined; Tables 14a and 14b show respectively the results obtained by hydrolysis only (14a) and by reduction, deslating and hydrolysis (14b) of samples of this material which had been partially purified by cellulose thin-layer chromatography. The variations between the two sets of figures were ascribed to losses of amino acids and small peptides, particularly those of an acidic or basic nature,

during the electrodialysis procedure which formed a part of the desalting process. However, the amount of proline had risen markedly.

A further series of experiments was carried out with a view to eliminating the errors introduced by desalting. The aim was to eliminate the necessity to desalt by electrodialysis by keeping the amount of lithium borohydride, and hence the amount of salt produced, to a minimum. This was done by using approximately ten times as much lithium borohydride as peptide material; Chibnall and Rees (1958), working with low molecular weight proteins, showed that an eight-fold molar excess of lithium borohydride was sufficient to reduce all ester linkages, and as the amino acid analysis of the unreduced material (Table 15a) suggested a reasonably high molecular weight ($>1,000$), the amount of borohydride used was clearly sufficient to bring about complete reduction. Commercial lithium borohydride was purified by the method of Chibnall and Rees (1958) and the tetrahydrofuran was dried by distillation from lithium aluminium hydride. The first attempt at reduction, omitting the electrodialysis stage, led to the analysis shown in Table 15b; the results suggest that reduction had not in fact occurred, presumably due to traces of moisture destroying the borohydride. The only apparent change was the decrease in the amount of threonine, due to base-catalysed degradation. A further reduction was therefore carried out, giving the results shown in Table 15c; in this case reduction did appear to have occurred satisfactorily. The results of these analyses still show marked variations, and it was thought possible that losses of amino acids might occur during the removal of borate by evaporation with methanolic

hydrochloric acid, since this provides the optimum conditions for the formation of amino acid esters. Fischer (1906) showed that of the ethyl esters of the common amino acids, those of glycine and alanine were the most volatile, while the diesters of glutamic and aspartic acids were the least volatile. The methyl ester of alanine boils at 38-42°C at 15 mm. Hg., while the diethyl ester of aspartic acid boils at 119-20° under the same pressure. Although under the conditions used these esters would tend to exist as their hydrochlorides, which are not volatile, there remains the possibility that some loss might occur; such a loss would affect glutamic and aspartic acids least, such that any reduction in the amount of, say, glutamic acid, by its conversion to proline, would tend to be minimised. The results in Tables 15a and 15c show that reduction appears to lead to an increase in the amount of proline. Of significance is the fact that the various materials which gave rise to proline on reduction were those which were most soluble in tetrahydrofuran, either because of prior treatment with phenyl isothiocyanate (Table 9), or because of their character (Tables 14b, 15c). No significant drops in the amounts of glutamic acid were observed, but a possible explanation of this has been mentioned above; the increases in the amounts of proline are large relative to the amounts of proline, but would correspond to only a small percentage decrease in the amounts of glutamic acid.

There are two possible explanations for the increase in the amount of proline: the first, which has already been mentioned, assumes the existence of an ester linkage via the

α -carboxyl group of a glutamic ester residue. Reduction of such an ester would lead to the formation of 2-amino-5-hydroxy-valeric acid, which under the conditions of hydrolysis would be expected to form some proline. No 2-amino-5-hydroxyvaleric acid was detected in the hydrolysates, and it seems unlikely that its conversion to proline would be complete, but it is possible that it is eluted from the column used in the analyses at the same time as serine, and the analyses of the reduced peptides did suggest a slight increase in the amounts of serine.

The second possible source of proline requires the presence of a glutamine end-group in the protein and in the peptides examined. No such group has yet been observed, but amino acid analyses generally involve acid hydrolysis, which would lead to de-amidation of the glutamine residue. Glutamine readily cyclises to form pyrrolidone-5-carboxylic acid, which under reducing conditions would form proline. Of these two possibilities, the first seems rather better substantiated by experimental evidence.

C-phycoerythrin was methanolysed by the method of Crespi et al. (1967) primarily to compare the chromatographic properties of the phycoerythrin produced with the pigment isolated from C-phycoerythrin by peptic digestion. In fact the two were found to behave similarly on the one system used - cellulose thin-layer plates using solvent C. Amino acid analysis of this phycoerythrin, however, produced some surprising results - Crespi et al. (1967) had deduced a structure for this material by nuclear magnetic resonance studies, which showed that it was amino acid-free chromophore.

The present work showed that in fact there was some peptide material present - albeit only a small amount. If it is assumed that the chromophore accounts for about 4 per cent by weight of the native protein, a figure which is now generally accepted (see e.g. Lemberg and Legge, 1949; Clendenning, 1954) and if the recovery is assumed to be around 40 per cent by weight, as Crespi et al. (1967) assumed, then the analysis results, shown in Table 13c, indicate that the weight of peptide represents about 2 per cent of the weight of the chromophore. Since the peptide contains a number of amino acids, Crespi et al. might well have dismissed the signals given by these residues as "noise" on their nuclear magnetic resonance spectra. The actual analysis of this peptide material (Table 13c) is of interest as it is similar in many respects to those of the chromopeptides obtained by enzymic digestions. One possible explanation is that a small amount of the chromophore - possibly located in a different environment within the molecule - has not been detached from the peptide chain, and this fragment has been carried along with the free chromophore.

The various peptides which have been examined - the fluorescent material from Pronase digestion of B-phycoerythrin, the coloured material obtained from peptic digestion of C-phyococyanin and the peptide obtained by methanolysis - appeared to show a common trend in composition. The analyses in fact suggest that none of these materials were homogeneous - in order to fit whole number ratios to the values obtained, the molecular weights would have to be very large; however, certain consistencies in the values are apparent, although some variations, almost certainly caused by experimental error,

did occur. The aspartic acid: threonine: serine: glutamic acid ratio of 1.0:1.1:0.9:0.9 suggests the presence of these four in equimolar quantities; the variations in the amounts of these amino acids on reduction has already been discussed. The valine:isoleucine:leucine:phenylalanine ratios also appear to be fairly constant at 3:3:2:1.5, again suggesting the presence of a hydrocarbon-like region of the protein molecule associated with the chromophore. The concept of such an environment for the prosthetic groups of biliproteins is supported by the fact that the heme groups of hemoglobin and myoglobin appear to be located in hydrophobic regions of the globin molecule. Wang et al. (1958) for example, suggested that the observed contrasting behaviour of hemoglobin and free heme towards carbon monoxide and cyanide ion respectively may be satisfactorily explained by postulating that the heme in hemoglobin is covered with hydrophobic side chains of the globin. It does not however appear possible to draw any further analogies with the heme groups of hemoglobin or myoglobin, since attachment of these groups is through the conjugated metal atom (Kendrew, 1960; Perutz, 1960), and there is no such metal atom known to be associated with the phycobilins. The porphyrin nucleus in mammalian cytochrome C is bound by two thioether linkages to two cysteine residues (Tuppy, 1958), but there is no evidence for the existence of such linkages in the biliproteins. A better analogy might be with the plant pigment phytochrome which has a biliviolin type chromophore (See Fig. 1; Siegelman, 1965), but there is as yet no evidence as to the nature of the chromophore-apoprotein linkage in this protein (see Crespi et al, 1968).

In conclusion, there seems little doubt that the C-phyococyanin chromophore, or at least one such chromophore, is attached to the apoprotein by an ester linkage (see Introduction). The results of the present work would suggest that this linkage involves the ω -carboxyl group of a glutamic acid residue in the protein chain. The point at which this linkage attaches to the chromophore has not yet been established; one possibility is that a lactam keto group in the chromophore enolises, and the resulting lactim hydroxy group then participates in the ester linkage. Confirmation of the nature of this linkage would require the isolation and purification of a chromopeptide containing as few amino acid residues as possible; ideally one would hope to be able to isolate the chromophore with only one residue still attached. The most promising approach to this problem appears to be through the use of enzymes, and the most promising starting material would seem to be the alcohol-soluble material obtained by the action of Pepsin on phyococyanin by the method of Lemberg (1928). Indeed it is somewhat surprising that Lemberg's results have been almost completely ignored for forty years. It is hoped that work at present being undertaken in this laboratory will confirm the nature of this linkage.

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Extensive reference was made to "Chemistry and Biochemistry of Plant Pigments" (T.W. Goodwin, ed.), Academic Press, London, 1965. Quotations from this book appear on page 1 (lines 1-12); page 5 (lines 20-29); page 6 (lines 1-5) and pages 38 and 39, section on Biosynthesis.